Improved Bivalent Miniantibodies, with Identical Avidity as Whole Antibodies, Produced by High Cell Density Fermentation of *Escherichia coli*

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The combination of single-chain Fv-fragments (scFv) with a C-terminal, flexible linking region followed by a designed or natural dimerization domain provides a versatile system for targeted association of functional fragments in the periplasmic space of *Escherichia coli*. For homodimerization *in vivo*, two scFv fragments with a C-terminal hinge followed by a helix-turn-helix motif form "miniantibodies" with significantly higher avidity than in the case of leucine zipper containing constructs. The favorable design probably results in an antiparallel four-helix bundle and brings the homodimer to the same avidity as the whole IgA antibody, from which the binding site was taken. The molecular weight of the bivalent miniantibody is almost the same as that of a monovalent Fab fragment. We report here a high-cell density fermentation of *E. coli* producing these miniantibodies and a work-up procedure suitable for large scale production. Without any need of subsequent chemical coupling *in vitro*, approximately 200 mg/l of functional dimeric miniantibodies can be directly obtained from the *E. coli* culture.

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Bivalency is an effective means for increasing the functional affinity of an antibody to a surface or a polymeric antigen[1-3]. Recently, we developed a strategy to assemble bivalent "miniantibodies" in the periplasm of *E. coli* using small dimerization domains[1], based on single-chain Fv fragments[4-6] connected to a hinge peptide and an amphipatic helix. The helix was either taken from a parallel coiled-coil helix of a leucine zipper[7,8] or from a single helix in a designed antiparallel four-helix bundle[9,10]. Surprisingly, we found that the bundle helix, when equipped with a peptide extension forming a disulfide bond, leads to miniantibodies with higher avidity than the leucine zipper design[1]. In this paper we report miniantibodies of still further improved performance, which are indistinguishable from whole antibodies in functional affinity.

For therapeutic and some diagnostic purposes, antibodies have to be available in amounts up to several grams. To demonstrate the feasibility of such a large scale production, we describe a high cell density fermentation procedure of the improved dimeric miniantibodies in *E. coli*. To obtain functional bivalent molecules, the intradomain disulfide bond in each of the variable domains, which is crucial for folding and stability[11], must be formed, V_H and V_L in the scFv portion must associate, and two scFv must dimerize *in vivo* with the help of the dimerization domain. For this assembly to occur in *E. coli*, the protein must be secreted into the periplasmic space. In order to understand more precisely the limitations of this process, we have undertaken a careful monitoring of the physiology of the cells during the secretion period of these foreign proteins in high cell density fermentations, and compared different work-up procedures. We report, as a result, fermentation and purification conditions, which might be of general utility in producing correctly folded disulfide-containing proteins in the periplasm of *E. coli*.

Results

Construction of improved miniantibodies. We have attached a helix-turn-helix module[10] to link two single-chain Fv fragments *in vivo* by formation of a (putative) four-helix-bundle. The model antibody was McPC603, which has a known crystal structure[12] and binds phosphocholine. Its functional expression by secretion has been previously examined in shake cultures for the Fv fragment[13], Fab fragment[14] and the single-chain Fv fragment[4].

In the present design, we have extended the single-chain Fv fragment by attaching the upper hinge region from mouse IgG3 and two identical helices from the antiparallel four-helix bundle design of Eisenberg and coworkers, in which the helices are separated by a turn[9,10] (Fig. 1A, B and C). In this case, the putative four-helix-bundle can be formed from two molecules, each contributing two helices. In previous experiments we had found that a single "bundle" helix per fragment would not lead to tetramers in significant amounts, but that mainly dimers in equilibrium with monomers were obtained. To shift the equilibrium more towards dimers, we constructed a (putative) four-helix bundle by providing two helices on each unit.

In ELISA assays, this newly constructed miniantibody gives a response indistinguishable from the whole IgA McPC603 (Fig. 2A). This suggests that a stable association of dimers has been obtained with a similar arrangement of binding sites as in the whole IgA (Fig. 1B). While the absolute response of the whole IgA at concentrations of signal-saturation is slightly higher, this may be due to its much larger molecular size, allowing more than one second antibody to bind and thus resulting in a proportionally increased ELISA signal, whose cause is independent of the functional affinity. However, the signals for the whole antibody McPC603 below saturation are nearly identical to those of the scdHLX miniantibody. This implies that at low concentrations more scdHLX than whole antibody is bound so that the functional affinity of the scdHLX under the conditions of the ELISA may be even higher than that of the whole antibody McPC603. Interestingly, the performance of the scdHLX miniantibody is also substantially better than that of miniantibodies based on leucine zipper fusions (Fig. 1C, Fig. 2). While the reasons for this difference are not entirely clear, it should be noted that this assay measures both the functional affinity (also called avidity[2,3]) and the fraction of molecules having two intact binding sites. All miniantibodies were affinity purified and standardized to the same molarity of binding sites, but any dissociation of the scZIP miniantibody or denaturation

A

construct	scFv-fragment	hinge	amphipatic helices
scFv	VH-linker-VL		
scZIP	VH-linker-VL-	PKPSTPPGSS-	RMKQLEDKVEELLSKNYHLENEVARLKKLVGER *gabcdefgabcdefgabcdefgabcdefgabc*
scdHLX	VH-linker-VL-	PKPSTPPGSS-	GELEELLKHLKELLKG-PRK-GELEELLKHLKELLKGEF *fgabcdefgabcdefg* *fgabcdefgabcdefg*

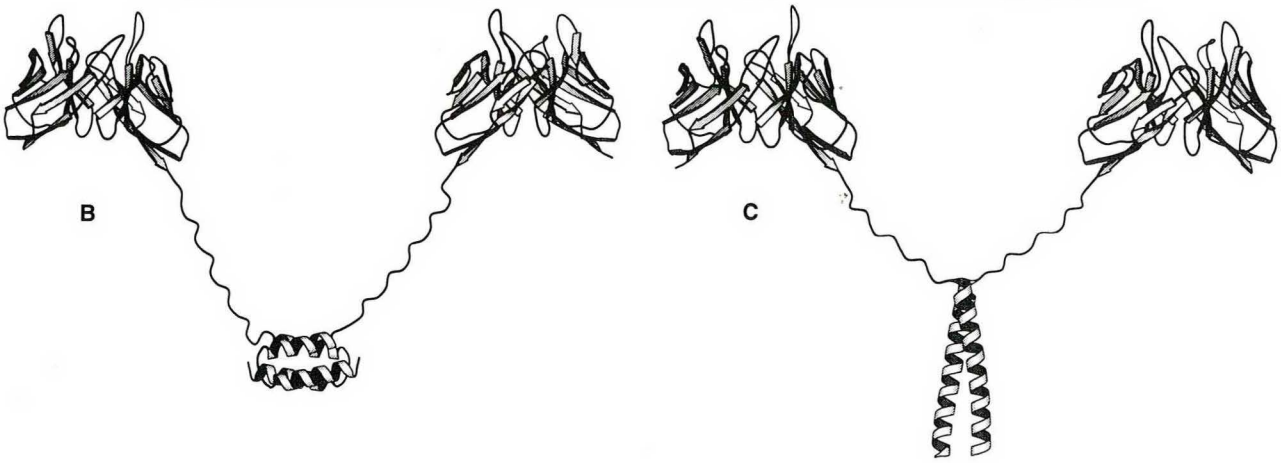


FIGURE 1. (A) The hinge region, which connects the C-terminus of the scFv fragment with the dimerization motif, was taken from mouse IgG3 (ref. 1). This sequence is responsible for flexibility and rotational freedom of the two scFv arms. It consists of the upper hinge region, which is the stretch of sequence between the end of C_H1 (the so-called "C_H1-elbow 2 bend") and the first cysteine linking the two heavy chains in the murine antibody[36,37]. The amphipathic helix as dimerization handle of the scZIP miniantibody was taken from the GCN4 zipper[7], with the characteristic heptad positions for coiled coils[38] given in italics. The sequence of the helix-turn-helix dimerization motif in construct scdHLX was designed by Eisenberg and coworkers[9,10], codons for the last two amino acids were added for cloning purposes. (B) The model of the miniantibody scdHLX was constructed as follows. The Fv fragment was taken from the crystal structure of the Fab fragment[12]. The $(Gly_4Ser)_3$ linker was was not modelled since recent NMR data suggest that it is a flexible structure with enhanced mobility[39]. The hinge region was taken as a polyproline II helix with $\phi = 78°$ and $\xi = 149°$. The 4-helix bundle was modelled based on the protein Rop[40], a natural 4-helix bundle. In a first step, the sequences were aligned at the hydrophobic positions. The leucines of the bundle helix (at position 3, 6, 10, 13; position a, d, a, d in Fig. 1A) now correspond to positions 12, 15, 19, 22 of Rop helix 1 and 38, 41, 45, 48 of Rop helix 2, respectively. The other residues were replaced according to the sequence using standard conformers, and the loop connecting the two helices (7 non-helical residues) was obtained by the loop search algorithm of A. Jones and co-workers as implemented in INSIGHT. Two additional residues at the end were added in standard α-helical geometry. A ribbon diagram drawn with the program MOLSCRIPT[41] is shown. (C) The Fv fragment and hinge region of miniantibody scZIP was modelled analogously to Figure 1B. The GCN4-zipper region was taken from the crystal structure[7].

TABLE 1. Comparison of fermentation runs.

Parameter	Run 1	Run 2	Run 3
Biomass concentration at the time of IPTG-addition (g/l)	20	14	17
Maximal biomass concentration (g/l)	58	47	50
Maximal concentration of total antibody[a] fragments (mg/l)	400	840	1035
Maximal concentration of active antibody[a] fragments (mg/l)	198	274	192
Maximal concentration of active antibody[b] fragments (mg/l)	78	54	96
Plasmid loss (% β-lactamase negative colonies 7 h after induction)	43	7	6

[a]After sonication. [b]After periplasmic extraction.

of one of the binding sites would lead to a loss of the bivalency gain in a fraction of the molecules, and thus lead to some molecules undetectable by functional ELISA[15].

If the coating density is varied, differences between scZIP and scdHLX become apparent (Fig. 2B and C). Because of the complexity of the system, it is not possible to quantitatively interpret these data in terms of a simple model[2]. However, the more pronounced increase of the response of scdHLX with coating density suggests a more effective accomodation to distant haptens. Also, the scattering of signal response was found to be higher for scZIP than for scdHLX in all ELISA experiments.

The dimeric nature of the two different miniantibodies scdHLX (held together by a putative four-helix bundle) and scZIP (held together by a leucine zipper) were tested by gel chromatography. Both miniantibodies were isolated as functional dimers on phosphocholine affinity chromatography, as evidenced by the fact that both proteins elute mostly as dimers on gel chromatography (Fig. 3). There is only an insignificant shoulder at the MW of the monomer in case of scdHLX and none for scZIP. Nevertheless, it is the scdHLX antibody which leads to a higher sensitivity in functional ELISA than the leucin zipper-based scZIP, by a factor of ten (Fig. 2A). The degradation of the original IgG3 hinge was found to be only marginal as evidenced by gel scanning and Western blots (data not shown).

High cell density cultivation (HCDC). Although a large number of proteins have been produced at industrial levels in the cytoplasm of *E. coli* in either soluble form or as inclusion bodies, the reported successes for secreted proteins is much lower. The problem lies in the fact that the secretion of some foreign proteins is a stress for *E. coli*, and the cell may respond with plasmid loss and in severe cases with genetic rearrangements of the plasmid[16]. Furthermore, in shake cultures characterized by low cell densities and an uncontrolled growth rate, a leakiness of the outer membrane is frequently observed[14].

A typical high cell density fermentation of *E. coli* RV308(pACK02sc-kan) is shown in Figure 4 (run 3 in Table 1). Until addition of IPTG, the cells grow exponentially in an almost balanced manner. This is indicated by a constant doubling time, a nearly constant nitrogen level in the culture medium, the smoothly accelerating rate of consumption of initial glucose, the smoothly varying kinetics of dissolved oxygen concentration and of the molar fraction of CO_2 and of O_2 in the exit gas (Fig. 4A and B). After addition of IPTG to induce miniantibody formation, the cells continued to grow in an almost unchanged manner for 4 hours. Even significant changes in the concentration of glucose did not enhance the formation of acetate, which is usually produced as the main by-product in *E. coli* cultures[17]. Only a continuous decrease of ammonia nitrogen in the culture medium indicated that metabolic imbalances started to occur immediately after IPTG-addition, and the cessation of steady state growth. A comparison of the kinetics of cell growth and formation of miniantibody fragments showed unequivocally that growth inhibition was due to the product itself (Fig. 4 B).

The maximum product level was reached four hours after IPTG addition, and the later increases of ammonia nitrogen and of acetate were consequences of the cessation of growth (Fig. 4). In some experiments (e.g. run 1, in Table 1), the optical density even decreased slightly after reaching a maximum, indicating some cell lysis at the very end of the run. It should be noted that the periplasmic leakiness observed in shake cultures with low cell densities and uncontrolled growth rates does not usually correspond to decreases in optical density. However, severe cell lysis of the same strain and a dramatic drop of optical density has been observed in shake cultures during expression of the single-chain F_v fragment and the related scdHLX miniantibody 3 hours after induction with IPTG.

Analysis of plasmid loss in the cell population as a function of time showed a high stability during growth before IPTG addition in all fermentation runs, but the results obtained after IPTG addition were not uniform. While almost no plasmid loss occurred until the end of fermentation runs 2 and 3, only half of the cells contained plasmids at the end of run 1 (Table 1). Antibiotics, which are present in these experiments, become ineffective after cessation of growth.

Efficiency of different protein extraction procedures. Table 1 also indicates that the preparation of periplasmic extracts was ineffective since only approximately 35% on average (distribution between 20–50%) of the active soluble miniantibodies was obtained, compared to the yield after sonication. This was confirmed by the result that a further 8–13% could be obtained by a second periplasmic extraction procedure of the remaining spheroblasts, and an additional 55–65% could be detected in functional ELISA after sonication of the twice extracted spheroblasts. Significant differences between independent preparations of periplasmic extracts indicate that this method is not suitable for the quantitative extraction of miniantibodies. To test wether this inclomplete release is due to the size of the dimeric miniantibody or the fermentation conditions, the content of monomeric scFv fragment and the miniantibodies scdHLX and scZIP in cellular fractions of shake cultures with low densities was jugded by immunoblotting (data not shown). In every case, even two-fold periplasmic extraction led only to a partial release for every kind of immunoglobulin tested. Within the accuracy of immunoblotting, expression yields of the monomeric scFv and the dimeric miniantibodies in several dilution series of soluble whole cell-extracts were similar. We can conclude, therefore, that the dimerization domains, which represent only 10–15% of the total molecular weight of miniantibodies, do not influence expression yields significantly. During fermentation, sonication was the method of choice for preparing and quantifying

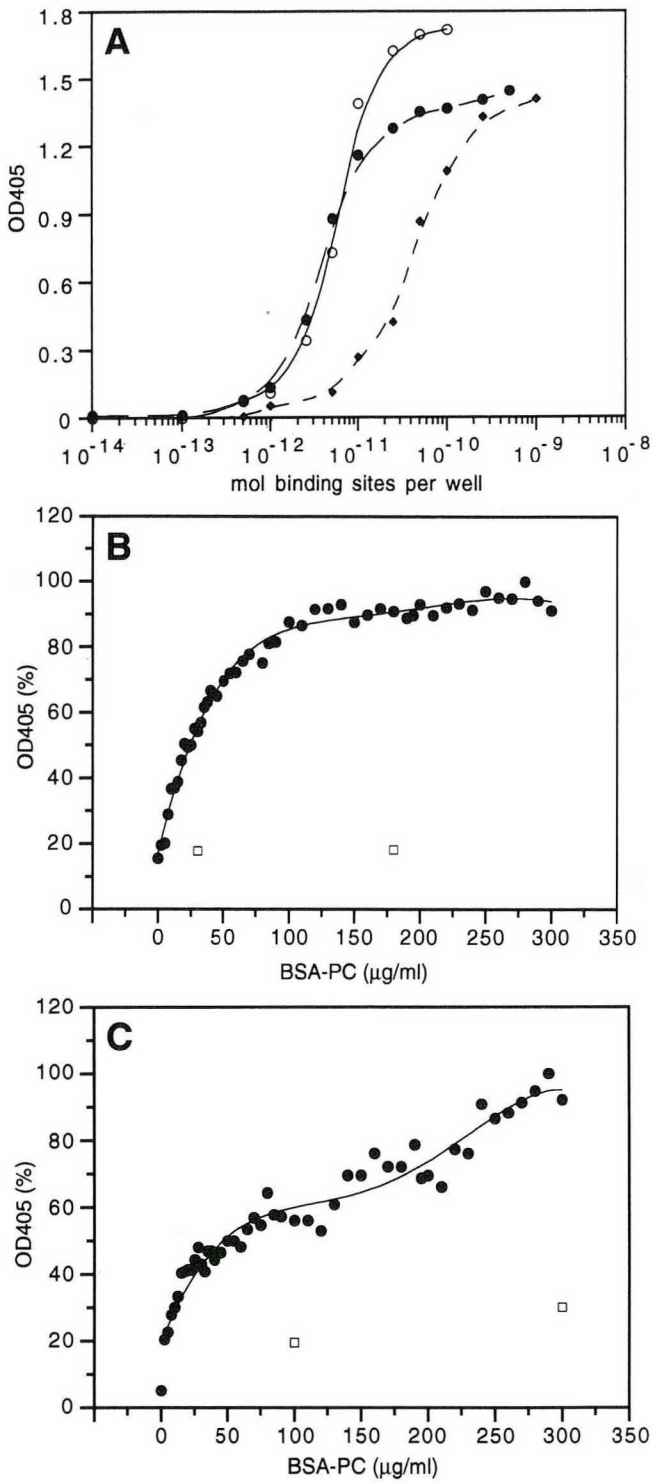


FIGURE 2. Functional ELISA of the miniantibody constructs scdHLX and scZIP. (A) Comparison of McPC603 (whole monomeric IgA, (○)) and the miniantibodies scdHLX (●) and scZIP (■). The ELISA wells were coated with BSA-PC (200 µg/ml), and the amount of antibody fragment per well (given as mol binding sites) is indicated. (B) Competitive coating ELISA of scdHLX. Coating of the ELISA plates was carried out with a total of 300 µg/ml of a mixture of BSA and BSA-PC. The concentration of the latter is given; the amount remaining to 300 µg/ml was BSA. The amount of miniantibody was 4×10^{-12} mol/well (antigen binding site). The squares denote samples, in which functional binding was inhibited by 1 mM soluble PC. The maximum signal (OD_{405}) for the given concentration range was set to 100%. (C) Competitive coating ELISA of scZIP. The experiment was exactly analogous to (B).

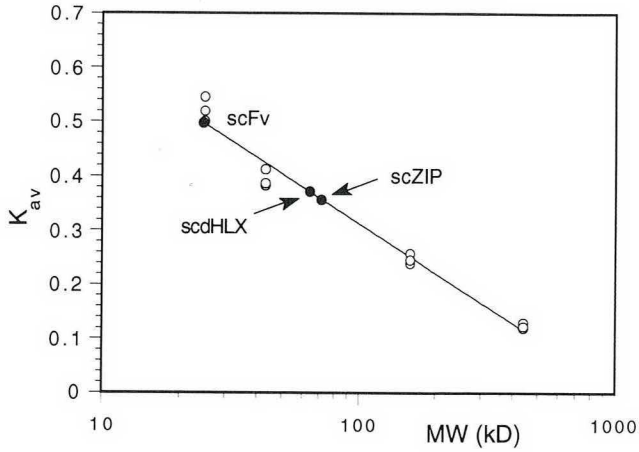


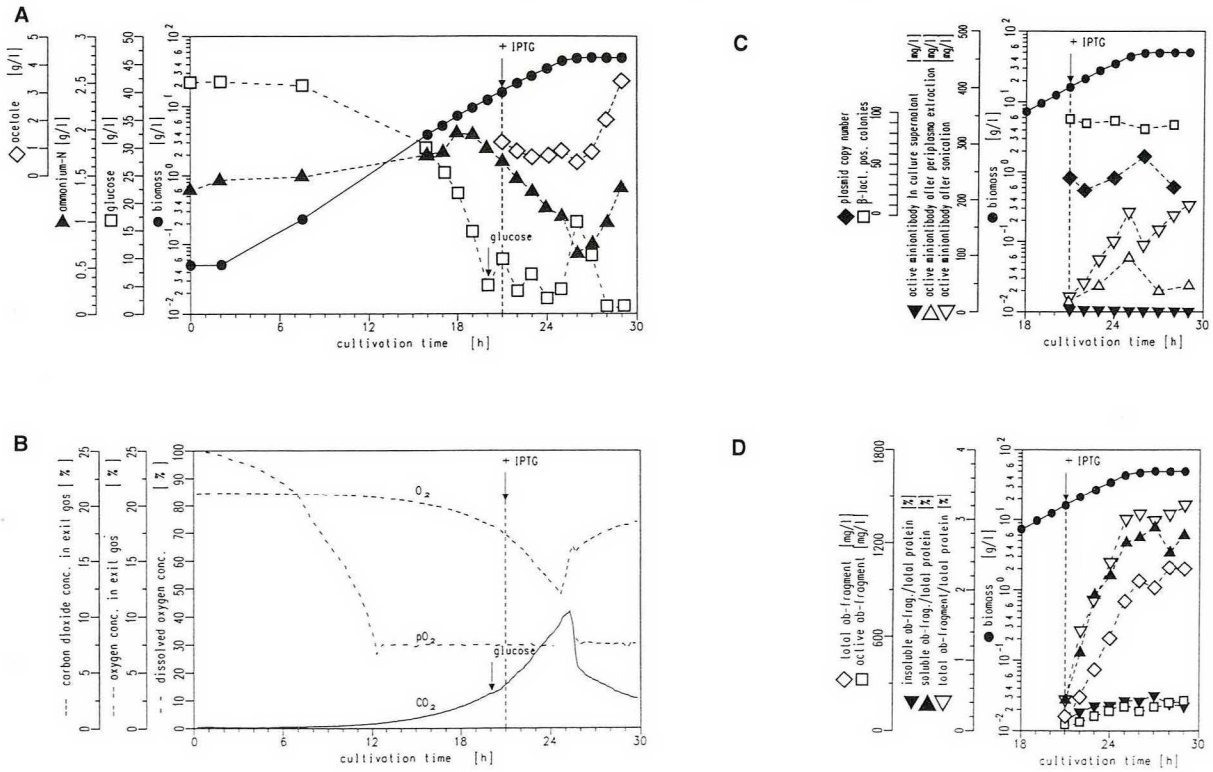
FIGURE 3. Size exclusion chromatography of the scFv fragment and the two miniantibody constructs, scdHLX (containing the putative four-helix-bundle) and scZIP (containing the leucine zipper of GCN4). The column used was Superdex 200 (Pharmacia), equilibrated and run in BBS, pH 8.0. Standards (○) were chymotrypsin (25 kD), ovalbumin (43 kD), aldolase (158 kD) and ferritin (440 kD).

soluble protein extracts independently of growth conditions or construct.

Interestingly, no significant amount of active miniantibody protein was detected in the medium during fermentation. Therefore, neither significant "secretion" into the medium, nor more likely cell lysis or permeability increase of the outer membrane is observed during the conditions of HCDC. This is in distinct contrast to shake cultures of the same fragments in a wide variety of different strains and different media (data not shown) and of Fv, scFv and Fab fragments in a similar vector in shake flasks[14]. We conclude that this leakiness is neither a particular property of some *E. coli* strains nor of a particular signal sequence, but rather is directly related to the growth physiology. During the balanced growth in a fermentor, essentially no leakiness was observed (Fig. 4C), although there is still insoluble miniantibody (Fig. 4D).

To eliminate erroneous quantitation of the miniantibodies in fermentation, protein binding to the ELISA plate non-specifically was excluded by working up a mock fermentation with plasmid-free *E. coli* RV308 under identical conditions (including IPTG-addition and sample treatment) as the production experiment with RV308(pACK02sc-kan). In the plasmid-free cells, only very low ELISA readings were obtained (0.1% of the real sample). Furthermore, in the real sample, 75–80% of the signal was inhibited by soluble phosphocholine. The remaining 20–25% of the sample signal might be due to miniantibody protein not bound via the PC binding site, and/or perhaps

FIGURE 4. High cell density cultivation (HCDC) of *Escherichia coli* RV308(pACK02kan). (A) Kinetics of the off-line data biomass (●), glucose (□), ammonia nitrogen (▲) and acetate (◇) during HCDC. (B) Kinetics of the on-line data pO_2, concentrations of CO_2 and O_2 in offgas during HCDC. (C) Functional miniantibody accumulation in culture supernatant (▼), after periplasmic extraction (△) and after sonication (▽) The plasmid stability was characterized by plasmid copy number (♦) and β-lactamase positive colonies (□) after addition of IPTG (1 mM) during HCDC. The concentration of active miniantibodies in the culture supernatant is very low (≤0.5 mg/l), but detectable. (D) Formation of soluble and insoluble miniantibodies after addition of IPTG (1 mM) during HCDC. All protein concentrations were determined from sonicated samples of cells. The concentration in mg/ml of total antibody protein (◇) was determined by gel scanning (error ± 10%) from the percentage of total antibody fragment per total protein (▽) and the total protein concentration[32]. In the same fashion, the distribution of miniantibodies between insoluble (▼) and soluble fractions (▲) was measured in comparison with total protein in these fractions. The concentration of active miniantibody protein in mg/ml (□) was determined from duplicate ELISAs as follows: Functional ELISAs were carried out as described in the Experimental Protocol, the functional protein determined from a standard curve with purified miniantibody, itself calibrated[34] by theoretical OD_{280}. The ELISA value was multiplied by a factor of 0.75, since it was found that only 75% of the reading is inhibitable by 1 mM soluble PC.



miniantibody aggregated in the work-up or ELISA procedure. In the calculation of active miniantibody, only the PC-inhibitable fraction was counted.

Yields of active and total miniantibody fragments. Similar maximal yields of active miniantibody protein (approximately 200 mg/l) were determined from three independent fermentation runs with RV308(pACK02sc-kan) (Table 1). Relatively high levels of basal expression (active antibody fragments reaching 0.2–0.3% of total *E. coli* protein, total antibody fragments reaching 0.4–0.7% of total *E. coli* protein) were found at the time of IPTG addition in every run. The increase of product (active antibody fragments reaching 0.6–1.2% of total *E. coli* protein, or total antibody protein reaching 2.2–3.8% of cell protein) stopped about 4–5 hours after induction. Product levels then remained constant or decreased slowly until the end of the fermentations. This shows that the wild-type *lac* promoter is somewhat leaky under the conditions of glucose feeding, but the low expression level before induction does not cause any metabolic problems nor does it lead to plasmid loss.

The miniantibody protein was also purified by affinity chromatography from sonicated cells (see Experimental Protocol). These results indicated purification yields of 60–75%, related to the ELISA values.

The distribution of total antibody fragments between soluble and insoluble protein fractions was quantified in sonicated samples after IPTG-addition (Fig. 4D). For this calculation, total cell protein was determined in the soluble and insoluble fraction (see Experimental Protocol), and the relative amount of antibody protein was estimated by gel scanning. From these data, 66–85% of the miniantibody was found in the soluble fraction and 15–34% in the insoluble fraction. This shows that not all molecules reach the correctly assembled state, but the cells apparently tolerate the insoluble protein better under the controlled growth conditions than in shake cultures.

Discussion

All natural antibodies are at least bivalent. By binding with two sites simultaneously to a solid surface, a higher functional affinity (avidity) is obtained. To a first approximation, this gain is inversely proportional to the maximal distance between the two binding sites, proportional to the two-dimensional epitope density on the surface and proportional to the binding constant of the monomeric fragment[2,3]. Corrections have to be made, however, for sites already occupied, the energetic cost of "bending" the antigen or the antibody and the nonideality of these processes close to a surface. In this paper, we present an improved bivalent miniantibody which is identical in terms of avidity as the whole antibody under the same conditions, but has only one third its size.

The design is based on a symmetric four-helix bundle, pioneered by Eisenberg, DeGrado and coworkers[9,10]. Each antigen binding site, present as a single-chain Fv fragment is connected, via a flexible hinge region, to a helix-turn-helix motif. This sequence (Fig. 1A) appears to be compatible with transport through the membrane, and it dimerizes in the periplasm, since completely functional dimeric molecules can be isolated directly. We cannot exclude that some of the protein is membrane associated, since the processed and soluble monomeric scFv fragments as well as dimeric miniantibodies are only released quantitatively during complete rupture of the cells.

The phosphocholine-binding antibody McPC603 (refs. 12, 18), with which the experiments were carried out, is only of moderate intrinsic affinity[18] (binding constant 1.6×10^5 M^{-1}). Monomeric fragment-hapten complexes of this antibody do not survive the stringent washing steps of a functional ELISA[1]. Since quantitation of the miniantibodies was carried out by functional ELISA, both binding sites are, therefore, intact, and our reported yields refer only to fully functional species.

Interestingly, the functional affinity (avidity) of the four-helix bundle construct is significantly higher than that containing the leucine zipper, a device which has also been used to alter quaternary structures[19-21]. Since association constants of the amphipathic helices used are not precisely known, we cannot rigorously rule out a correlation of the binding properties with the intrinsic dimerization constants of the two different structures. We could not find, however, any positive influence of a C-terminal, covalently connecting disulfide bond on the functional affinity of the leucine zipper-based miniantibody[1].

One possible explanation of the better performance of the four-helix-bundle than the leucine zipper is its greater stability in dilute solutions, since any decrease in the number of functionally bivalent molecules would be accounted for in our experiments as a lower functional affinity. Another contributing factor might be a larger distance spanned between the two binding sites by the antiparallel arrangement of helices or interference between the folding of the leucine zipper and the hinge region. In addition, it appears that there is a greater tendency of the leucine-zipper based antibodies to bind non-specifically to the ELISA-plate at concentrations higher than 1×10^{-11} mol binding site per well (data not shown) and greater scattering of the ELISA data (Fig. 2C). Since a greater portion of the hydrophobic surface of each amphipathic helix would be shielded in a 4-helix bundle than in a coiled-coil structure made up from two helices, there may be fewer side reactions such as aggregation or nonspecific surface binding in the 4-helix bundle. Therefore, the four-helix bundle is our preferred motif for dimerization.

The resulting miniantibody scdHLX is suitable for high cell density fermentations, and the functional bivalent protein can be purified in good yield. It is stable against sonification and the prolonged contact with proteases in crude extracts during purification and quantification procedures. Despite the relatively long and flexible hinge regions (Fig. 1), proteolytic digestion has only been observed in small fractions of the molecules during storage for several days above 4°C.

While the w. t. *lac* promotor was found to be somewhat leaky under the HCDC conditions and some product is observed before induction, this background expression does not noticeably disturb the metabolic balance of the cells, which grow with a constant doubling time. Only *after* induction, there can be some plasmid loss in a few runs. To minimize plasmid loss, kanamycin resistance is preferred, since the antibiotic cannot be destroyed by lysed cells, as can β-lactam antibiotics from leaked β-lactamase. For yet unknown reasons, it appears that background expression is higher in the kan^R amp^R plasmid than in the amp^R plasmid (L. Ge and A. Plückthun, unpublished observations).

The miniantibodies described here can be obtained directly in dimeric form from *E. coli*, and the host-vector system was suitable for fermentations up to a biomass of 60 g dry cells per liter. Penetration of solid tumor tissue is dependent on size[22], and the present bivalent fragments are the MW of a monovalent Fab fragment. The improved miniantibodies, combining small size with high avidity and rapid clearance of unbound molecules *in vivo* (Haunschildt et al., in preparation), have therefore properties desirable in tumor immunology[22,23], and other clinical applications.

Experimental Protocol

E. coli strains. The prototrophic *E. coli* K12 strain RV308 (*lac*74-*gal*ISII::OP308*str*A)[24] harboring pACK02sc-kan (see vector construction) was used in high cell density cultivation (HCDC). Unspecific binding of *E. coli* proteins in ELISA was determined from a mock fermentation of RV308 not harboring any plasmid (see below). A *tonA* derivative of RV308 (strain 25F2) was also used as a host for expression of humanized Fab′ fragments[25].

Construction of vector pACK02sc-kan. Recombinant DNA techniques used were based on those of Sambrook et al.[26]. The vector pACK02sc-kan is based on pASK-lisc[4] and encodes a single-chain Fv antibody (scFv) construct[11] of the phosphocholine-binding antibody McPC603 (ref. 18). The modules for expression of the association domains, encoding the upper hinge region of mouse IgG3 (ref. 1) and the amphipathic helices[7,9,10] (Fig. 1 A) were synthesized as a combination of four oligonucleotides. The singular EcoRI site, into which the gene cassettes were ligated, is at the very end of the V_L domain (.....ELKRANGEF) encoding Glu-Phe. In addition to the ampicillin resistance, the vector carries a 1540 bp EcoRI-fragment encoding the kanamycin resistance inserted between the f1-origin and the β-lactamase gene, which both remain functional. For sensitive and highly specific detection of scFv fragment and miniantibodies by immunoblotting, a shortened version (Knappik and Plückthun, unpublished) of the FLAG-peptide[27] was inserted between signal sequence and N-terminus of the V_H domain.

Media for HCDC. The composition and preparation of the media is based on published data[28], but several modifications were made. The initial glucose/mineral salt media contained per liter: 13.3 g KH_2PO_4, 1.7 g citric acid, 60 mg Fe(III)citrate, 10 ml trace metal solution (consisting of 1.5 g/l $MnCl_2 \cdot 4H_2O$, 0.3 g/l H_3BO_3, 0.25 g/l $Na_2MoO_4 \cdot 2H_2O$, 0.25 g/l $CoCl_2 \cdot 6H_2O$, 0.15 g/l $CuCl_2 \cdot 2H_2O$ and 0.84 g/l EDTA, Na_2-salt$\cdot 2H_2O$), 8 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 1 g $(NH_4)_2HPO_4$ and 2.7 g $MgSO_4 \cdot 7H_2O$. Initial glucose concentrations were 10 g/l for shaking cultures (inoculants) and 40 g/l for production cultures in the fermentor. Media for RV308 harboring pACK02sc-kan were supplemented with both ampicillin (50 mg/l) and kanamycin (25 mg/l). To prevent precipitation, the initial medium was prepared according to a special protocol: KH_2PO_4, Fe(III)-citrate, trace metal solution, $Zn(CH_3COO)_2 \cdot 2H_2O$ and $(NH_4)_2HPO_4$ were sequentially added as solutions to about half of the final volume. After autoclaving, sterile solutions of glucose, $MgSO_4 \cdot 7H_2O$, ampicillin and kanamycin were added after prior adjustment of the pH to 6.8 with 12.5% (v/v) ammonia. Sterile distilled water was added to adjust the initial volume to 0.1 L in flasks for starter cultures and to 8 L minus inoculum volume in the fermentor. Feeding solutions were 12.5% (v/v) ammonia, the antifoam reagent Ucolub N115 and 70% (w/v) glucose.

High cell density cultivation (HCDC) and controls. Cultivations were carried out in fed-batch mode in a 10-L-fermentor BIOSTAT ED10 equipped with DCU (Digital Measurement and Control System) and Micro-MFCS (Multi Fermentor Control System) from B. Braun Biotech International and exhaust gas analyzer from Hartmann & Braun AG (Melsungen and Frankfurt, FRG). The reactor was operated at a temperature of 26°C, a pressure of 0.15 MPa and a pH of 6.8 with a gas flow rate of 10 l/min. An exponentially grown starter culture was used as inoculum. During cultivation, the dissolved oxygen concentration was kept at $\geq 20\%$ of saturation by a pO_2/agitation rate control loop and a pO_2/gas flow ratio control loop. The second control loop was initiated after reaching the maximum agitation rate (1500 rpm), and at this point the incoming air was enriched with pure oxygen. Aqueous ammonia served both for pH control and as nitrogen source. Foaming was suppressed by controlled supply of the antifoam reagent Ucolub N115. In the fed-batch phase, the feeding rate of glucose was adjusted manually to keep the concentration of glucose between 0.5 and 14 g/l. For induction of miniantibody synthesis, IPTG (isopropyl-β-D-thiogalactopyranoside, Promega, USA) was added to a final concentration of 1 mM. During cultivation, temperature, pressure, pH, agitation rate, total gas flow rate, air flow rate, oxygen flow rate, and mol fractions of oxygen and carbon dioxide in the exhaust gas were measured and recorded continuously. Optical density at 550 nm, cell dry weight, glucose, ammonia nitrogen and acetate were determined as a function of fermentation time as described previously[28].

Stability of pACK02sc-kan in *E. coli* RV308 during HCDC. Both the average plasmid copy number and the fraction of plasmid harboring cells in the population were estimated as a function of fermentation time. The percentage of plasmid harboring cells in the cell population was determined from the number of β-lactamase positive colonies in the following way[29]. Culture samples were diluted, spread on LB-agar in Petri dishes and incubated at 34°C overnight. After brief air drying, they were overlaid with 5.6 ml of a mixture containing 3 ml 1% (w/v) agarose in 50 mM potassium phosphate buffer pH 7.0, 1.5 ml 2% (w/v) starch dissolved in 50 mM potassium phosphate buffer pH 7.0, 100 μl aqueous solution of 2.3% (w/v) iodine and 60% (w/v) potassium iodide as well as 1 ml 0.15% (w/v) ampicillin. Halos developed around β-lactamase positive colonies within several minutes, whereas the surroundings of β-lactamase negative colonies remained dark blue. Plasmid copy numbers were estimated by densitometric scanning (Cybertech CS1 gel documentation System, Cybertech, Berlin) of PstI-linearized plasmid DNA after agarose gel electrophoresis and ethidium bromide staining, based on the assumption that plasmid DNA was extracted quantitatively[30]. The copy number was calculated from the total amount of plasmid DNA in the fermentation samples (estimated with four calibration standards of known DNA concentration, themselves quantitated by measuring OD_{260}), the molecular mass of one plasmid, the number of cells in the sample aliquot as determined from dried biomass aliquots, and an assumed dry weight of an *E. coli* cell of $2.8 \cdot 10^{-13}$ g (ref. 31). The calibration standards were prepared by linearizing purified DNA of pACK02sc-kan with PstI.

Sample preparation for determination of active and total antibody fragment amounts. Immediately before addition of IPTG (t = 0) and after every hour, 10 ml samples were withdrawn from the culture. The samples were centrifuged (10,000x*g*, 4°C, 4 min). The cell-free supernatant was used for determination of the product released into the culture medium. The cell pellet was resuspended with cold (4°C) BBS-EDTA buffer (200 mM borate, adjusted with NaOH to pH 8.0, 160 mM NaCl, 1 mM EDTA) to OD_{550} of 5 and 2, respectively. Ten ml of the cell suspension adjusted to OD_{550} of 5 were sonicated (Techpan UDM10, Poland; 2 x 1 min, 4°C, 100 W), and 4 ml of these disintegrated cells were used for the determination of total protein concentration[32] (BSA was used as protein standard) as well as for PAGE (see below). From the remaining sample, insoluble protein was separated by centrifugation (10000x*g*, 4°C, 10 min). The supernatant contained the soluble antibody fragments. The active antibody fragments were determined by a functional ELISA (see below). The extracts of soluble periplasmic protein were prepared by vortexing the cell resuspension adjusted to $OD_{550} = 2$ for 90 min at 4°C and collecting the supernatant after a further centrifugation step. For determination of immunoglobulin content in cell-fractions by immunoblotting, whole cell extracts were prepared by threefold disruption in a French press homogenizer[11].

Functional ELISA. The ELISA was carried out essentially as described[1] on Maxisorp microtiter plates (Nunc) coated with phosphocholine-derivatized BSA. Using these rigorous washing procedures, the OD_{405} signal was almost exclusively produced by intact bivalent miniantibodies (see Results), based on the gain in binding observed for all miniantibody constructs in relation to the low efficiency of binding of monomeric scFv-fragments[1]. After purification[1], the respective miniantibody protein was used as a standard in ELISA. The protein concentration of the purified standard was calculated from the OD_{280} value, corrected for possible interference by nucleic acids[33], using the calculated[34] extinction coefficient of $OD_{280} = 1.95$ at 1 mg/ml.

Controls. The specific binding capacity of miniantibody protein to phosphocholine-BSA adsorbed on a microtiter plate is the basis of the ELISA signal (OD_{405}). To demonstrate specific binding, a phosphocholine solution (in BBS-EDTA buffer; 5 mM final concentration) or an aliquot volume of BBS-EDTA buffer were added to the samples in the wells before the ELISA procedure was carried out. The OD_{405} not inhibitable by phosphocholine should then be a measure of nonspecifically bound protein and/or aggregated antibody fragments. At two different dilutions, it was found that about 25% of the ELISA signal is not inhibitable by 5 mM PC. The yield of active miniantibody protein based on the ELISA values was compared to the amount of active antibody protein isolated and purified from defined sample volumes by phosphocholine affinity chromatography[4]. For this purpose, 30 ml of a sonicated and sterile-filtered sample from a resuspension ($OD_{550} = 5$) of cells harvested 4 h after IPTG addition were used in chromatography. The concentration of antibody protein in the eluate was determined as described for the ELISA standard.

Protein gel electrophoresis, immunoblotting and gel scanning. Discontinuous SDS-PAGE under reducing conditions was carried out as described[35] in 12% polyacrylamide gels. Coomassie brilliant blue stained gels were scanned in an Ultroscan XL gel scanner (Pharmacia LKB) with the Gelscan XL 2400 software program. In immunoblots of cellular fractions[11], processed immunoglobulins were identified with murine antibodies against the N-terminal FLAG-peptide[27] (Eastman Kodak, New Haven).

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References

1. Pack, P. and Plückthun, A. 1992. Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in *Escherichia coli*. Biochemistry **31**:1579–1584.
2. Crothers, D. M. and Metzger, H. 1972. The influence of polyvalency on the binding properties of antibodies. Immunochemistry **9**:341–357.
3. Karush, F. 1978. The affinity of antibody: range, variability, and the role of multivalence, pp. 85–116. *In:* Immunoglobulins. Litman, G. W. and Good, R. A. (Eds.). Plenum Publishing Corp., NY and London.
4. Glockshuber, R., Malia, M., Pfitzinger, I. and Plückthun, A. 1990. A comparison of strategies to stabilize immunoglobulin Fv-fragments. Biochemistry **29**:1362–1367.
5. Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S.-M., Lee, T., Pope, S. H., Riordan, G. S. and Whitlow, M. 1988. Single-chain antigen-binding proteins. Science **242**:423–426.
6. Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R. and Oppermann, H. 1988. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85**:5879–5883.
7. O'Shea, E. K., Klemm, J. D., Kim, P. S. and Alber, T. 1991. X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. Science **254**:539–545.
8. O'Shea, E. K., Rutkowski, R. and Kim, P. S. 1989. Evidence that the leucine zipper is a coiled coil. Science **243**:538–542.
9. Eisenberg, D., Wilcox, W., Eshita, S. M., Pryciak, P. M., Ho, S. P. and DeGrado, W. F. 1986. The design, synthesis, and crystallization of an alpha-

helical peptide. Proteins **1:**16–22.
10. Ho, S. P. and DeGrado, W. F. 1987. Design of a 4-helix bundle protein: synthesis of peptides which self-associate into a helical protein. J. Am. Chem. Soc. **109:**6751–6758.
11. Glockshuber, R., Schmidt, T. and Plückthun, A. 1992. The disulfide bonds in antibody variable domains: effects on stability, folding *in vitro*, and functional expression in *Escherichia coli*. Biochemistry **31:**1270–1279.
12. Satow, Y., Cohen, G. H., Padlan, E. A. and Davies, D. R. 1986. Phosphocholine binding immunoglobulin Fab McPC603. An X-ray diffraction study at 2.7Å. J. Mol. Biol. **190:**593–604.
13. Skerra, A. and Plückthun, A. 1988. Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. Science **240:**1038–1041.
14. Plückthun, A. and Skerra, A. 1989. Expression of functional Fv and Fab fragments in *Escherichia coli*. Meth. Enzymol. **178:**497–515.
15. Pack, P., Knappik, A., Krebber, C. and Plückthun, A. 1992. Mono- and bivalent antibody fragments produced in *E. coli* : binding properties and folding *in vivo*, pp. 10–13. *In:* ACS Conference Proceedings Series, Harnessing Biotechnology for the 21st Century. Ladisch, M. R. and Bose, A. (Eds.) ACS, Washington, DC.
16. Wülfing, C. and Plückthun, A. 1993. A versatile and highly repressible *E. coli* expression system based on invertible promoters. Gene. *In press*.
17. Riesenberg, D. 1991. High-cell-density cultivation of *Escherichia coli*. Current Opinion in Biotechnol. **2:**380–384.
18. Metzger, H., Chesebro, B., Hadler, N. M., Lee, J. and Otchin, N. 1971. Modification of immunoglobulin combining sites, pp. 253–267. *In:* Progress in immunology: Proceedings of the 1st Congress of Immunology. Amos, B. (Ed.). Academic Press, New York.
19. Blondel, A. and Bedouelle, H. 1991. Engineering the quaternary structure of an exported protein with a leucine zipper. Prot. Engineering **4:**457–461.
20. Hu, J. C., O'Shea, E. K., Kim P. S. and Sauer, R. T. 1990. Sequence requirements for coiled coils: analysis with λ-repressor-GCN4 leucine zipper fusions. Science **250:**1400–1403.
21. Kostelny, S. A., Cole, M. S. and Tso, Y. J. 1992. Formation of a bispecific antibody by the use of leucine zippers. J. Immunol. **148:**1547–1553.
22. Thomas, G. D., Chappell, M. J., Dykes, P. W., Ramsden, D. B., Godfrey, K. R., Ellis, J. R. M. and Bradwell, A. R. 1989. Effect of dose, molecular size, affinity, and protein uptake of antibody or ligand: a biomathematical model. Cancer Res. **49:**3290–3296.
23. Huston, J. S., McCartney, J. S., Tai, M.-S., Mottola-Hartshorn, C., Jin, D., Warren, F., Keck, P. and Opperman, H. 1993. Medical applications of single-chain antibodies. Intern. Rev. of Immunol. *In press*.
24. Maurer, R., Meyer, B. J. and Ptashne, M. 1980. Gene regulation at the right operator (O_R) of bacteriophage λ-O_R3 and autogenous negative control by repressor. J. Mol. Biol. **139:**147–161.
25. Carter, P., Kelley, R. F., Rodrigues, M. L., Snedecor, B., Covarrubias, M., Velligan, M. D., Wong, W. L. T., Rowland, A. M., Kotts, C.E., Carver, M. E., Yang, M., Bourell, J. H., Shepard, H. M. and Henner, D. 1992. High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. Bio/Technology **10:**163–167.
26. Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. Hopp, T. P, Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, C. P., Urdal, D. L. and Conlon, P. J. 1988. A short polypeptide marker sequence useful for recombinant protein identification and purification. Bio/Technology **6:**1204–1210.
28. Riesenberg, D., Menzel, K., Schulz, V., Schumann, K., Veith, G., Zuber, G. and Knorre, W. A. 1990. High cell density fermentation of recombinant *Escherichia coli* expressing human interferon alpha 1. Appl. Microbiol. Biotechnol. **34:**77–82.
29. Löser, C. 1990. Stabilitätsverhalten eines rekombinanten ColE1-Plasmid-derivates mit Ampicillin-Resistenzmarker und Human-Interferon-α-I-Gen in *Escherichia coli*. PhD-Thesis. Technical University Dresden, Fed. Rep. of Germany.
30. Bignon, C. and Martin, P.-M. 1992. Improved sequencing of "mini-prep" double-stranded templates using a multiwell microplate system. Biotechniques **13:**104–106.
31. Neidhardt, F. C. 1987. Chemical composition of *Escherichia coli*. 3-6. *In: Escherichia* and *Salmonella typhimurium*. Cellular and Molecular Biology. F. C. Neidhardt (Ed. in chief). ASM Washington, D.C.
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193:**265–275.
33. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Meth. Enzymol. **3:**447–454.
34. Gill, S. T. and von Hippel, P. H. 1989. Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. **182:**319–326.
35. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
36. Burton, D. R. 1985. Immunoglobulin G: functional sites. Mol. Immunol. **22:**161–182.
37. Dangl, J. L., Wensel, T. G., Morrison, S. L., Stryer, L., Herzenberg, L. A. and Oi, V. T. 1988. Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. EMBO J. **7:**1989–1994.
38. Lupas, A., Van Dyke, M. and Stock, J. 1991. Predicting coiled coils from protein sequences. Science **252:**1162–1164.
39. Freund, C., Ross, A., Guth, B., Plückthun, A. and Holak, T. 1993. Characterization of the linker peptide of the single-chain Fv fragment of an antibody by NMR spectroscopy. FEBS Lett. **320:**97–100.
40. Banner, D. W., Kokkinidis, M. and Tsernoglou, D. 1987. Structure of the ColE1 Rop protein at 1.7Å resolution. J. Mol. Biol. **196:**657–675.
41. Kraulis, P. J. 1991. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. **24:**946–950.