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Tetravalent Miniantibodies with High Avidity Assembling in *Escherichia coli*

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We have designed tetravalent miniantibodies assembling in the periplasm of *Escherichia coli*. They are based on single-chain Fv fragments, connected *via* a flexible hinge to an amphipathic helix which tetramerizes the molecule. The amphipathic helix is derived from the coiled coil helix of the transcription factor GCN4, in which all hydrophobic *a* positions of every heptad repeat have been exchanged to leucine and all *d* positions to isoleucine. Gel filtration shows tetramer assembly of the miniantibody even at low concentrations. As expected, the functional affinity (avidity) of the tetravalent miniantibody is higher in ELISA and BIAcore measurements than that of the bivalent construct and the gain is dependent on surface epitope density.

Keywords: antibodies; single-chain Fv fragments; leucine zipper; B/A core; multivalency

The low intrinsic affinity of antibodies produced early after immunization is compensated for very effectively by means of multivalency. This natural strategy of polymerization of binding sites, for instance in the form of a decavalent immunoglobulin M, leads to an increase in functional affinity (avidity) toward multimeric antigens, such as viral or bacterial surface epitopes, of several orders of magnitude (Devey & Steward, 1988). The gain in stability of antibody-antigen complexes depends on the thermodynamic affinity of a single binding site (intrinsic affinity), the number of binding sites per molecule and the number of epitopes within reach, and is strongly influenced by geometric factors (Crothers & Metzger, 1971; Kaufman & Jain, 1992).

We previously utilized the benefits of bivalency by designing miniantibodies (Pack & Plückthun, 1992). The model with which the design was tested was the well characterized phosphorylcholine-binding antibody McPC603 (Satow *et al*., 1986) in the form of a single-chain Fv fragment (Glockshuber *et al*., 1990), connected to an association motif *via* a flexible hinge region. Two different motifs have previously been tested: parallel associating coiled coil helices (O'Shea *et al*., 1991) and antiparallel associating helices of a four-helix bundle design (Ho & DeGrado, 1987). In the latter case, when using only one bundle helix per fragment, nevertheless mostly dimers were obtained. Only a small fraction of the affinity-purified protein behaved consistently with tetramers in ultracentrifugation studies (Pack & Plückthun, 1992). Dimeric miniantibodies with further improved binding characteristics could be formed using a helix-turn-helix motif instead of a single helix for association. The resulting scdHLX miniantibody is expressed in yields of several hundred milligrams per liter culture in high cell density fermentation of *Escherichia coli* (Pack *et al*., 1993).

Here we report a new application of helical association domains to produce tetravalent miniantibodies assembling *in vivo* in the periplasm of *E. coli*. It is based on the observation of P. Kim, T. Alber and co-workers that the simultaneous change of four amino acid residues in heptad position *a* and four amino acid residues in heptad position *d* of the leucine zipper dimerization domain of the GCN4 protein (Figure 1) can result in the formation of highly stable trimeric and tetrameric synthetic peptides (Harbury *et al*., 1993). Changing of the hydrophobic residues in position *a* to leucine and the

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Abbreviations used: BIAcore™, biosensor device; Ig, immunoglobulin; BSA, bovine serum albumin; BSA-PC, conjugate from BSA and PC; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; PC, phosphorylcholine; RU, resonance units; scFv, single-chain Fv fragment; scTETRAZIP, fusion protein from modified tetramerizing leucine zipper and single-chain Fv fragment; scZIP, fusion protein from leucine zipper and single-chain Fv fragment; scdHLX, fusion protein from helix-turn-helix peptide and single chain Fv fragment.

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construct	**scFv-fragment**	hinge	helix
scZIP	**VH-linker-VL**	- PKPSTPPGSS -	R**M**KQ**L**EDK**V**EE**L**LSK**N**YH**L**ENE**V**AR**L**KKL**V**GER
scTETRAZIP	**VH-linker-VL**	- PKPSTPPGSS -	R**L**KQ**I**EDK**L**EE**I**LSK**L**YH**I**ENE**L**AR**I**KKL**L**GER
heptad position			g**a**bc**d**efg**a**bc**d**efg**a**bc**d**efg**a**bc**d**efg**a**

Figure 1. Sequence comparison of the unmodified GCN4-zipper (amino acid residues 249 to 281 of the GCN4 protein) used as an association handle in the construct scZIP and zipper modified according to Harbury *et al*. (1993), used in the construct scTETRAZIP. Positions exchanged in the heptads of the modified zipper part are indicated in bold type.

conserved leucine residues in position *d* to isoleucine leads to a parallel four-helix complex as revealed by X-ray crystal structure analysis and deduced from re-equilibration experiments of peptides carrying additional cysteine residues at the N or C terminus. According to the findings of Harbury *et al*. (1993) the coiled coil packing of the dimer cannot accommodate the side-chains of isoleucine at position *d* pointing into the hydrophobic core, or at least, the tetrameric packing is energetically favored. Deviations from two-stranded association have also been seen with several other related peptides, in which the nature of the hydrophobic *a* and *d* positions in the typical heptad repeat (Lupas *et al*., 1991) of coiled coil sequences was altered (Lovejoy *et al*., 1993; Zhu *et al*., 1993).

The tetramerizing protein was constructed and expressed in *E. coli* in analogy to the dimeric miniantibodies (Pack & Plückthun, 1992) by fusing the modified GCN4 zipper to the single-chain Fv fragment *via* a hinge region. Here we describe the successful expression and purification of this tetrameric miniantibody (named scTETRAZIP) and compare its functional behavior with a miniantibody dimerized with the unmodified GCN4 zipper (named scZIP). Despite the similarity of the individual domains of the constructs the protein yield of the tetrameric miniantibody was initially drastically lower (1.5 μg/l per A_{550} unit) than for the dimer-miniantibody. This problem was circumvented by exchanging three amino acid residues in the heavy chain which are important for efficient folding (Knappik & Plückthun, 1995); additionally four amino-terminal amino acid residues were added for the detection with the anti-FLAG antibody (Knappik & Plückthun, 1994). The parallel, tetrameric nature of this protein may increase its sensitivity to inefficient folding, as the whole tetramer might be lost if just one of the scFv units fails to fold and starts to aggregate cooperatively. These mutations (Knappik & Plückthun, 1995) as well as slight modifications in the production procedure (Figure 2) increased the yield of functional tetrameric protein 100-fold to 0.2 mg/l per A_{550} unit in shake flasks. To keep the binding domains identical for functional comparisons, the stabilizing mutations and the short FLAG-epitope were also introduced into the dimeric construct scZIP. Starting from a higher yield, the relative increase was smaller, and finally 0.8 mg/l per A_{550} unit were obtained in shake flasks.

Size exclusion chromatography was used to analyze the oligomerization state of the miniantibodies (Figure 2). This was to test whether the tetramerization seen with peptides in ultracentrifugation studies at concentrations from 20 to 200 μM (Harbury *et al*., 1993) is also seen in proteins purified from *E. coli* at a concentration as low as 0.5 μM. The highly symmetrical peak at the expected molecular weight of 130 kDa in the chromatogram demonstrates that a stable tetramer is purified. In comparison, the miniantibody scZIP elutes at half the molecular weight, corresponding to dimers, while the scFv fragment elutes at the expected monomeric molecular weight. All these proteins give single peaks in the elution profile, indicating a stable oligomerization for the zipper as well as for the tetra-zipper miniantibody and no unspecific aggregation of the scFv fragment.

The binding characteristics of the dimeric and

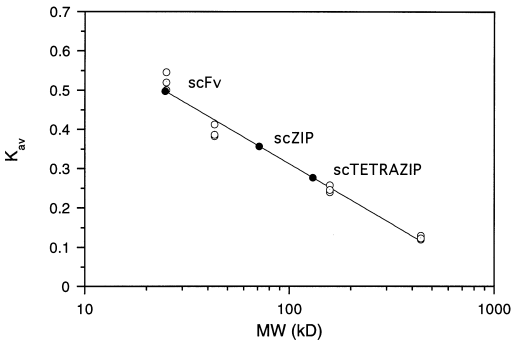

Figure 2. Size exclusion chromatography of the scFv fragment and the 2 miniantibody constructs, scZIP (containing the GCN4-zipper) and scTETRAZIP (containing the modified leucine zipper) at 1 μM protein. The column used was Superdex-200 (Pharmacia), equilibrated and run in borate-buffered saline (BBS: 200 mM borate/NaOH (pH 8.0), 160 mM NaCl). Standards (open circles) were chymotrypsin (25 kDa), ovalbumin (43 kDa), aldolase (158 kDa) and ferritin (440 kDa). The expression and purification of the miniantibodies largely followed published procedures (Pack & Plückthun, 1992, and references cited therein). However, a more robust *E. coli* strain derived from RV308 allowing longer induction times was used and cells were disrupted by sonification. During phosphorylcholine affinity chromatography of the tetrameric, but not of the dimeric, miniantibody, a protein co-purified, subsequently identified as GroEL (P. Pack, R. Zahn & A. Plückthun, unpublished observation), which necessitated an additional size exclusion chromatography purification step on a Superdex-200 column.

tetrameric miniantibody were compared with surface plasmon resonance using the BIAcore instrument (Jönsson *et al*., 1991) and ELISA. For both methods, phosphorylcholine coupled to bovine serum albumin (BSA-PC) was used for coating the chip or plate. Under these conditions, binding of a monomeric scFv is not detectable by ELISA due to the relatively weak intrinsic binding constant of $1.6 \times 10^5\ M^{-1}$ (Metzger *et al*., 1971, Glockshuber *et al*., 1990). The fast dissociation rate of the monomeric complex of 10 to $38\ s^{-1}$ (Goetze & Richards, 1977) does not allow one to observe the on- and off-rates directly with the BIAcore instrument, but a steady state is reached quickly. The bivalent complex formed by bivalent miniantibodies is more stable and overcomes the unfavorable dissociation rate of the monomeric scFv (Pack & Plückthun, 1992).

The BIAcore sensograms at three different antigen immobilization densities and various miniantibody concentrations of the dimeric and the tetrameric miniantibody are compared in Figure 3. The different binding behavior of the dimer and the tetramer can clearly be seen and binding constants can be estimated. The problem of multivalency has been addressed with the BIAcore method (Ito & Kurosawa, 1993). It should be kept in mind, however, that a quantitative evaluation is made difficult by high coating densities required for obtaining dimeric binding, bringing the on-rates in the neighborhood of mass-transport control and causing rebinding in the dissociation phase. Furthermore, individual components from the multiphasic kinetics observed are hard to separate.

To prevent mass transport limitation the flow rate was chosen to be in a range where the response was shown to be flow-rate-independent. This required a significantly higher flow rate for the tetrameric protein than for the dimer. Rebinding, which is often a problem for the evaluation of *intrinsic* affinities, is in our case an interesting property of the molecules to be compared and it accounts partially for the avidity effect.

At the lowest immobilization density the dimeric miniantibody shows mostly monovalent binding as indicated by immediately reaching a steady state (Figure 3A). The reaction is too fast to be kinetically resolved by the instrument. Hence only the equilibrium constant can be estimated by plotting signal *versus* concentration. The value found was about $10^6\ M^{-1}$. Two facts indicate that dimeric binding occurs at higher coating densities. First, the association kinetics change, which is best explained by a slower reaction overlaying the fast monomeric kinetics. Second, the relative remaining signal at time points after the monomeric dissociation is complete (e.g. 100 seconds) increases with the coating density (Figure 3C). Rebinding is likely to account for a large part of this signal and this rebinding has to be dimeric as it is not observed with the scFv monomer. Although this experiment does not rule out dimeric binding to a single BSA-PC molecule, the dependence on coating density makes it more likely that the miniantibody bridges two BSA-PC molecules. The second, slow phase during the association and dissociation of the dimer is likely to be due to bivalent binding with strict stereochemical requirements.

The tetramer never shows an immediate plateau and thus always binds at least bivalently. Apparently, even at the lowest antigen concentration, BSA-PC molecules can be bridged or bound bivalently, probably because of the larger distance spanned by the tetramer. Additionally, there are many more combinations of two binding sites out of the four, of which one may fit favorably. The overall reaction of the tetramer has a slower on-rate but also a much slower off-rate, leading to a notably higher affinity than the dimer, despite being at the border of mass-transport limitation, as evidenced by measurements at various flow rates (data not shown). A closer look at Figure 3 reveals that this reaction deviates slightly from monophasic kinetics. The difference between the tetramer and the dimer in the rate constants is critically dependent on the conditions and the time point of comparison. At low coating densities and high tetramer concentrations, multiple binding is not favored and rebinding is hindered due to the surface being covered by the miniantibody itself (Figure 3A). Thus, in the latter case, the tetramer kinetics have a significant portion of fast kinetics, which is best explained by monomeric binding. At high coating densities and low concentrations, the dissociation of the tetramer is reduced to zero. This effect is also seen in the second part of the dissociation of the dimer (Figure 3C). At all coating densities and concentrations, even when taking the different masses of scZIP and scTETRAZIP into account, the tetramer reaches much higher response units, which also reflects a higher avidity.

To get further insight into the binding properties of the tetramer, the running buffer in the dissociation phase was supplemented with PC. This causes an immediate elution (Figure 3), demonstrating that the binding is entirely specific. The fast and efficient interception of surface binding by soluble PC also shows that there must be a high fluctuation of the individual binding sites, which makes the tetramer elute with the enormously fast intrinsic off-rate when soluble PC is added.

To obtain a more quantitative comparison of the binding properties the sensograms were analyzed using a pseudo-first-order kinetic model implemented in the vendor software (Karlsson *et al*., 1991), which gives an idea of the overall binding properties using the linear range of the relevant plot (Table 1). Since this model is not valid for the observed kinetics, which are more complicated as easily seen in the case of the dimer, a biphasic association and dissociation model was used to fit the data obtained with the medium coating density surface (Figure 3B). Using this model the fast components in the association phase are relatively similar (Table 1). The striking difference between the dimeric and tetrameric miniantibody is, according to this model, based on the difference in the dissociation rate and the different contributions (amplitudes) of the fast and slow kinetics to the signal. In the case of the dimer

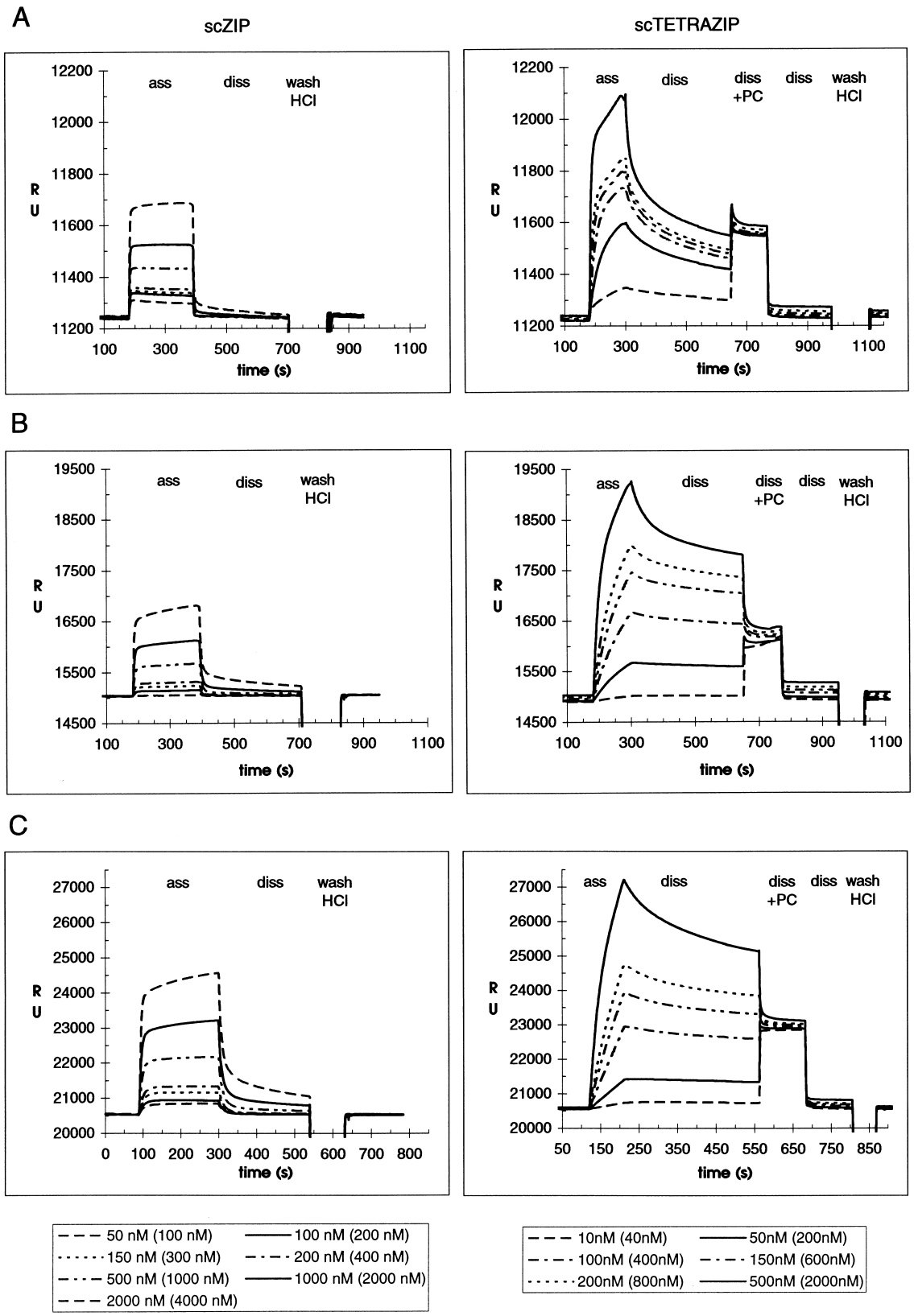

Figure 3. Comparison of BIAcore runs of the dimeric and tetrameric miniantibody at different coating densities and increasing concentrations of the miniantibodies. Coating densities were: A, 1270 BIAcore resonance units (RU); B, 5000 RU; C, 10,000 RU. Phosphorylcholine was coupled to BSA as described (Pack & Plückthun, 1992) and the derivatized BSA was coupled to the sensor chip (CM5 research grade) using *N*-ethyl-*N*'-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) amide coupling. The running buffer was BBS, which was also used as dialysis buffer and dilution buffer for the samples. The flow rate was 8 μl/min for the scZIP and 16 μl/min for the scTETRAZIP measurements. For the additional washing step, running buffer was supplemented with PC at a concentration of 20 mM in runs A and B and 5 mM in run C. The elevated signal level during the washing with PC reflects the refractive index of this solution. HCl (20 mM) was used for chip regeneration. In control experiments performed with underivatized BSA or without coating, binding and the bulk effect were negligible.

Table 1

Kinetic parameters† for the dimeric and tetrameric miniantibody derived from the BIAcore measurements

Coating density (resonance units)	scZIP				scTETRAZIP			
	$k_{ass} \times 10^{-5}$ (M^{-1} s^{-1})		$k_{diss} \times 10^{2}$ (s^{-1})		$k_{ass} \times 10^{-5}$ (M^{-1} s^{-1})		$k_{diss} \times 10^{2}$ (s^{-1})	
1270	k_{ass}‡	—	k_{diss}‡	—	k_{ass}§	3	k_{diss}§	0.2–0.3
5000	k_{ass}§	1	k_{diss}§	1–2	k_{ass}§	0.8	k_{diss}§	0.02–0.09
	k_{ass} fast‖	0.8	k_{diss} fast‖	17–19	k_{ass} fast‖	1	$k_{dss\ fast}$‖	1–2
	k_{asss} slow‖	0.03	k_{diss} slow‖	0.3–0.6	k_{asss} slow‖	0.06	k_{diss} slow‖	0.02–0.05
10,000	k_{ass}§	1	k_{diss}§	1–2	k_{ass}§	0.5	k_{diss}§	0.01–0.09

† Goetze & Richards (1977) obtained with soluble phosphorylcholine and the whole McPC603 antibody an intrinsic association rate constant of 9.5 to 38×10^5 M^{-1} s^{-1} and a dissociation rate constant of 10 to 38 s^{-1} from NMR measurements.

‡ This reaction was too fast to be kinetically resolved, therefore only the equilibrium constant was obtained from the plateau level (see the text).

§ Monophasic evaluation: the slope of the dR/dt *versus* R plots yields k_{obs}, which was plotted against the concentration of soluble antibody to obtain k_{ass}, and $\ln(R_1 / R_n)$ was plotted *versus* time to obtain k_{diss} using the vendor software as described by Karlsson *et al*. (1991).

‖ Biphasic evaluation: 2 independent pseudo-first-order phases describing reactions of the type $A + B \rightleftarrows AB$ were used. The assumptions were: [A] is constant, $[B] = R_{max} - R$ and [AB] equals the resonance signal R. Integration of the rate equation:

$$\frac{dR}{dt} = k_{ass}[A](R_{max} - R) - k_{diss}R$$

gives an expression for the resonance-signal as a function of time:

$$R = \frac{k_{ass}[A]R_{max}}{k_{ass}[A] + k_{diss}}(1 - e^{-(k_{ass}[A] + k_{diss})t}).$$

Thus, the total expression used in fitting is of the form:

$$R = C1(1 - e^{-k_{obs1}t}) + C2(1 - e^{-k_{obs2}t}) + C3,$$

where $C1$, $C2$, k_{obs1}, k_{obs2} were the parameters fitted, and $C3$ was the fixed baseline. An analogous fit was used for the dissociation phase. A Marquardt–Levenberg algorithm implemented in the program Origin was used for fitting. The association constant was obtained from the plot k_{obs} *versus* concentration.

the amplitude ratio of the fast to the slow kinetics ranges from 1.7 to 3.9, whereas the same ratio for the tetramer ranges from 0.06 to 0.45. During the dissociation phase the difference in this ratio is even higher. We propose that this is an indication of the fraction of molecules displaying monovalent *versus* multivalent binding.

The dimeric and tetrameric miniantibodies were also compared in their binding properties in a competition ELISA. In this assay increasing amounts of the zipper and tetrazipper miniantibodies were added to a constant amount of TEPC15 antibody (Figure 4A), a related IgA which recognizes the same antigen (Perlmutter *et al*., 1984). The second, enzyme-linked antibody detected the constant α domain without cross-reaction. The displacing effect of the tetramer clearly exceeds the competition of the dimeric miniantibody, of which about a tenfold higher concentration is required for the same reduction in binding of the IgA. This is another validation that the avidity of the tetrameric molecule is increased.

In a direct comparison of ELISA signals obtained with increasing amounts of the dimeric and tetrameric miniantibodies, a sevenfold higher concentration of the scZIP molecule is needed to obtain the same response (Figure 4B). Since in this case the observed signal is mediated by a secondary antibody directed against the variable domains, part of the sensitivity increase may be due to more secondary antibodies binding to the twofold larger scTETRAZIP. However, the different shape of the response curve is inconsistent with this being the only cause of the increased sensitivity of the scTETRAZIP. Rather, there must also be an increase in avidity, which is consistent with the competition ELISA and BIAcore data (see above).

The gain in stability of scTETRAZIP-antigen complexes in ELISA is also seen in comparing the response as a function of antigen coating density (Figure 4C and D). A steeper increase in the response is seen for the scTETRAZIP than for the scZIP, when unspecific binding is accounted for (Figure 4C and D). This suggests that more molecules find a suitable second binding partner in the case of the tetramer than the dimer at low coating densities. Furthermore, a further binding site may become involved in the case of the scTETRAZIP at high coating density.

Since the inhibition of binding by soluble hapten is more quantitative and the reproducibility of the signal is better in the case of the scTETRAZIP than the dimeric scZIP, we conclude that the tetramerization of the zipper sequences leads to a better shielding of the hydrophobic core, resulting in fewer hydrophobic and unspecific interactions than with the GCN4 zipper. This is very reminiscent of the comparison of the antiparallel four-helix bundles with the dimeric coiled-coil helices as dimerization motifs (Pack *et al*., 1993). Additionally, crystallographic data reveal that the hydrophobic surface buried by association is 900 $Å^2$ per helix in the parallel dimer, but 1640 $Å^2$ per helix in the tetrameric, altered version of the GCN4 zipper (O'Shea *et al*., 1991; Harbury *et al*., 1993). We suggest that four-helix arrangements are generally superior to dimeric coiled coil helices because the latter expose too much of the hydrophobic surface in the dimeric state. Hence single helices of the type

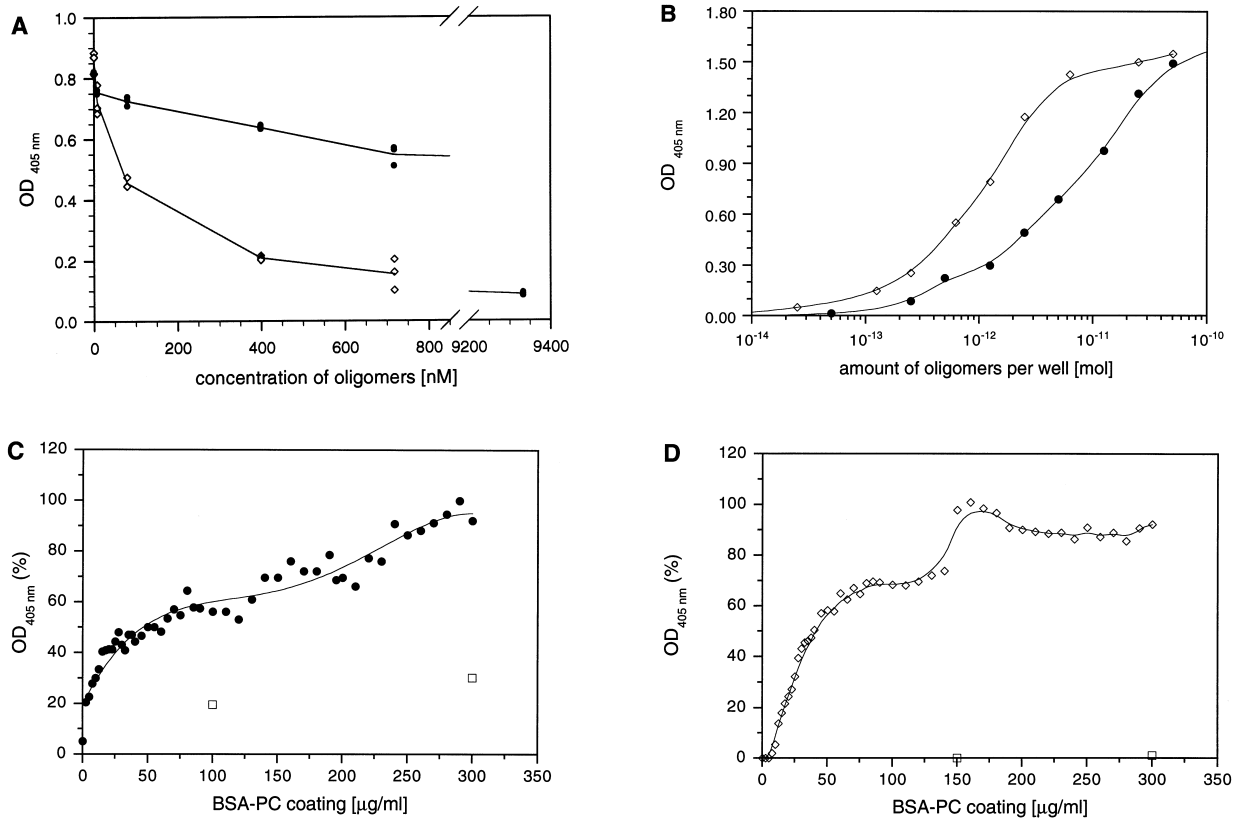

Figure 4. A, Competition ELISA detecting the binding of the mouse IgA TEPC15, which is displaced by either the dimeric miniantibody (●) or the tetrameric miniantibody (◇). Microtiter plates were coated with 200 μg / ml BSA-PC, blocked with 3% (w / v) skim milk powder and washed with BBS. After incubating the antibody mixture for 1 h and washing with PBS-Tween TEPC15, binding was detected with a peroxidase coupled anti-α mouse serum (Sigma). TEPC15, purchased as ascites (Sigma) was reduced, alkylated (Goetzl & Metzger, 1970) and affinity purified like the miniantibodies. B, Functional ELISA of the miniantibody constructs scZIP and scTETRAZIP. Comparison of different concentrations of the miniantibodies was carried out at constant hapten density. The ELISA wells were coated with the hapten carrier BSA-PC (200 μg / ml), and the amount of antibody fragment per well (given as mol molecules / well) is indicated (◇, scTETRAZIP, ●, scZIP). A rabbit polyclonal serum against the McPC603 variable domains and as the second antibody an anti-rabbit-IgG serum was used. C, scTETRAZIP; and D, scZIP analyzed in a competitive coating ELISA (Pack *et al*., 1992). 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 sites). Open squares give the signal in the presence of 1 mM PC. Binding was detected as in B. The maximum signal (A_{405}) for the given concentration range was set to 100%. Inhibition of binding by soluble PC was 100% for scTETRAZIP but only 70 to 81% for scZIP. Therefore, a constant signal (about 25% of the maximum) is due to unspecific adsorption of scZIP to the plate and should be viewed as a baseline. Only the values above this level reflect functional binding and should be used in comparison with scTETRAZIP.

described here are suitable for tetramers and helix-turn-helix modules for dimers.

We have demonstrated a method to produce tetravalent scTETRAZIP miniantibodies, which have the molecular weight of bivalent $F(ab')_2$ fragments. They exhibit enhanced binding compared to the bivalent miniantibody scZIP as demonstrated by BIAcore and ELISA measurements. The effect of multivalency depends on the antigen density. By altering the hinge region, the antibody might be fine-tuned to a given biological problem. The design of our miniantibody may be useful in contexts where extremely high avidity is important or multiple binding is essential, like receptor cross-linking, yet the molecular weight should remain small. The general design principle should be useful to assemble a wide range of molecular complexes.

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References

Crothers, D. M. & Metzger, H. (1972). The influence of polyvalency on the binding properties of antibodies. *Immunochemistry*, **9**, 341–357.

Devey, M. E. & Steward, M. W. (1988). The role of antibody affinity in the performance of solid phase assays. In *ELISA and Other Solid Phase Immunoassays* (Kemeny, D. M. & Challacombe, S. J., eds), pp. 135-153, John Wiley & Sons Ltd, London.

Glockshuber, R., Malia, M., Pfitzinger, I. & Pückthun, A. (1990). A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry* **29**, 1362–1367.

Goetze, A. M. & Richards, J. (1977). Structure-function relations in phosphorylcholine-binding mouse myeloma proteins. *Proc. Nat. Acad. Sci., U.S.A.* **74**, 2109–2112.

Goetzl, E. J. & Metzger, H. (1970). Affinity labeling of a mouse myeloma protein which binds nitrophenyl ligands. Kinetics of labeling and isolation of a labeled peptide. *Biochemistry,* **9**, 1267–1278.

Harbury, P. B., Zhang, T., Kim, P. S. & Alber, T. (1993). A switch between two-, three- and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science,* **262**, 1401–1407.

Ho, S. P. & DeGrado, W. F. (1987). Design of a 4-helix bundle protein: synthesis of peptides which self-associate into a helical protein. *J. Amer. Chem. Soc.* **109**, 6751–6758.

Ito, W. & Kurosawa, Y. (1993). Development of an artificial antibody system with. multiple valency using an Fv fragment fused to a fragment of protein A. *J. Biol. Chem.* **268**, 20668–20675.

Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfas, S., Persson, B., Roos, H., Rönnberg, I., Sjölander, S., Stenberg, E., Stahlberg, R., Urbaniczky, C., Östlin, H. & Malmqvist, M. (1991). Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *BioTechniques,* **11**, 620–627.

Karlsson, R., Michaelsson, A. & Mattsson, L. (1991). Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. *J. Immunol. Methods,* **145**, 229–240.

Kaufman, E. N. & Jain, R. K. (1992). Effect of bivalent interaction upon apparent antibody affinity: experimental confirmation of theory using fluorescence photobleaching and implications for antibody binding assays. *Cancer Res.* **52**, 4157–4167.

Knappik, A. & Plückthun, A. (1994). An improved tag based on the FLAG peptide for the detection and purification of antibody fragments in *Escherichia coli*. *BioTechniques,* **17**, 754–761.

Knappik, A. & Plückthun, A. (1995). Engineered turns of a recombinant antibody improve its *in vivo* folding. *Protein Eng.*, in the press.

Lovejoy, B., Choe, S., Cascio, D., McRorie, D. K., DeGrado, W. F. & Eisenberg, D. (1993). Crystal structure of a synthetic triple-stranded α-helical bundle. *Science,* **259**, 1288–1293.

Lupas, A., Van Dyke, M. & Stock, J (1991). Predicting coiled coils from protein sequences. *Science,* **252**, 1162–1164.

Metzger, H., Chesebro, B., Hadler, N. M., Lee, J. & Otchin, N. (1971). Modification of immunoglobulin combining sites. In *Progress in Immunology: Proceedings of the 1st Congress of Immunology* (Amos, B., ed.), pp. 253–267, Academic Press, New York.

O'Shea, E. K., Klemm, J. D., Kim, P. S. & Alber, T. (1991). X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science,* **254**, 539–545.

Pack, P. & 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.

Pack, P., Knappik, A., Krebber, C. & Plückthun, A. (1992). Mono- and bivalent antibody fragments produced in *E. coli*: binding properties and folding *in vivo*. In *ACS Conference Proceedings Series, Harnessing Biotechnology for the 21st Century* (Ladisch, M. R. & Bose, A., eds), pp. 10–13, Amer. Chem. Soc., Washington DC.

Pack, P., Kujau, M., Schroeckh, V., Knüpfer, U., Wenderoth, R., Riesenberg, D. & Plückthun, A. (1993). Improved bivalent miniantibodies with identical avidity as whole antibodies, produced by high cell density fermentation of *Escherichia coli*. *Biotechnology,* **11**, 1271–1277.

Perlmutter, R. M., Crews, S. T., Douglas, R., Soerensen G., Johnson, N., Nivera, N., Gearhart, P. J. & Hood, L. (1984) The generation of diversity in phosphorylcholine-binding antibodies. *Advan. Immunol.* **35**, 1–37.

Satow, Y., Cohen, G. H., Padlan, E. A. & Davies, D. R. (1986). Phosphocholine binding immunoglobulin Fab McPC603. An X-ray diffraction study at 2.7 Å. *J. Mol. Biol.* **190**, 593–604.

Zhu, B.-Y., Zhou, N. E., Kay, C. M. & Hodges, R. S. (1993). Packing and hydrophobicity effects on protein folding and stability: effects of β-branched amino acids, valine and isoleucine, on the formation and stability of two-stranded α-helical coiled coils/leucine zippers. *Protein Sci.* **2**, 383–394.

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