Mono- and Bivalent Antibody Fragments Produced in E. coli: Binding Properties and Folding in vivo.

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We have designed dimeric antibody fragments of minimal size that assemble in E. coli and show an increase in avidity approaching a whole antibody. They are based on single-chain Fv 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 four-helix bundle design or a leucine zipper, optionally extended with a short cysteine containing peptide. To investigate the folding and assembly process of antibody fragments in E. coli, co-expression experiments with proline cis-trans-isomerase and disulfide isomerase were carried out. These folding steps do not appear to be limiting the folding process in E. coli.

Recent advances in the bacterial expression technology of antibodies (Plückthun, 1991), PCR cloning of antibody libraries (Orlandi et al., 1989) and library screening with the phage display technology (Marks et al., 1991) are decisive steps along a path to antibodies based almost entirely on molecular biology and biotechnology. This account will address the question of which antibody fragments are compatible with functional expression in E. coli, and by what folding mechanism they achieve the native state.

The bacterial expression of antibodies in the native state was first developed for the Fv fragment of the antibody (the non-covalent heterodimer of the variable domains V_L and V_H; Skerra and Plückthun, 1988) and the F_2b fragment (the first two domains of the heavy chain V_HC and the complete light chain V_LCL; Better et al., 1988). In this approach, the principles of antibody secretion and folding in the eukaryotic cell are partially mimicked by secreting both chains into the periplasm of the same E. coli cell. Therefore, each of the domains can act as a folding template for the other, and the disulfide bonds can form. Without the disulfide bonds in the variable domains, no functional product can be obtained in E. coli in any antigen binding fragment tested (Glockshuber et al., 1992, Skerra and Plückthun, 1991).

In order to increase the stability of the Fv fragment, the V_H and V_L domain can be linked covalently in vivo by two strategies, which are compatible with functional expression and secretion: (i) by engineering of an intermolecular disulfide bond (Glockshuber et al., 1990) and (ii) by connecting the two domains by a genetically encoded peptide linker to give a so-called single-chain Fv fragment (scFv) (Glockshuber et al., 1990; Huston et al. 1991). In the native expression approach, both possible designs (V_H-linker-V_L and V_L-linker V_H) have been used. Conflicting results have been obtained about the relative yields of the two designs when used on the same antibody. While Anand et al. (1991) observed dramatic differences, Knappik and Plückthun (unpublished results) found the yields to be very similar.

From Monovalent to Bivalent Fragments

All the antibody fragments so far reported as expressible in the native state in E. coli have been monovalent. While F_2b' fragments produced in E. coli have been linked chemically to (F_2b')_2 fragments after purification (Carter et al., 1992), we wished to investigate the question, whether an in vivo dimerization of antibody fragments is possible. Furthermore, our goal was to make these bivalent fragments as small as possible while achieving a maximal increase in avidity (Crothers and Metzger, 1972).

For this reason, we designed dimerizing single-chain Fv fragments, which we call miniantibodies (Fig. 1). As a model system, the phosphorylcholine antibody McPC603...
(Perlmutter et al., 1984) was used. Two alternative molecular designs have been tested (Pack and Plückthun, 1992). In the first, the single chain fragment (VH-linker-VL) was fused to the flexible upper hinge region of mouse IgG3. This was followed by one helix taken from the the 4-helix bundle designed by Eisenberg et al. (1986). This helix was either taken as such (construct scHLX) or extended by a small hydrophilic peptide ending in a cysteine residue (scHLXc) in order to covalently link two helices. Ultracentrifugation measurements are consistent with a dimer formation in vivo. From the arrangement of the charged residues on the helix, an antiparallel association is anticipated.

In the other molecular design, instead of the helix from the 4-helix-bundle design, a helix from a parallel coiled-coil structure was used (O'Shea et al., 1991). Specifically, we fused the leucine zipper peptide from the yeast transcription factor GCN4 to the scFv fragment. Again, the helix was either taken as such (scZIP) or extended with a short peptide ending in a cysteine (scZIPc).

The functionality of the fragments was investigated in several different ways. All miniantibodies can be purified by hapten affinity chromatography, illustrating that the antigen binding site forms correctly in E. coli. The covalently linked miniantibodies show dimer bands on a non-reducing PAGE. In ultracentrifugation measurements, all non-covalently linked and covalently linked miniantibodies give evidence of molecular weights compatible with dimers. Only in the case of the non-covalent antiparallel helices, there is a very small amount of faster sedimenting material, perhaps consisting of tetramers. Probably the non-covalent 4-helix-bundle is not stable enough to allow persistent tetramer formation under these conditions. The linking peptide may prevent

Fig. 1: (A) Molecular model of the human antibody KOL (B): Molecular model of the dimeric scHLXc miniantibody constructs derived from the single-chain Fv fragment of the mouse antibody McPC603 (Satow et al., 1986). The hinge region was modelled according to a polyproline-II helix with \( \phi = -78^\circ \) and \( \psi = 149^\circ \). A standard \( \alpha \)-helix with \( \phi = -57^\circ \) and \( \psi = -47^\circ \) was used for the amphipilic helix. The cys-tail peptide is presumably disordered, and the structure drawn should be taken only as a guide to the topology. (C): Molecular model of the scZIPc miniantibody construct. The parameters are identical as in A, except that a parallel coiled coil with about a quarter turn of the superhelix was modeled for the leucine zipper part (O'Shea et al., 1991).
tetramer formation in the case of the covalently linked variant, so that dimers are the predominant species in both cases.

Most importantly, however, all the bivalent miniantibodies show the desired gain in "avidity", an empirical measure of the increased apparent binding constant to polymeric or surface-bound antigen. The best performance was observed for the covalently linked antiparallel helix construct, whose binding properties are almost identical to those of the complete IgA, although the bivalent miniantibody has the MW of only one Fab fragment. The observed differences in avidity between the different miniantibodies (Pack and Plückthun, 1992) may be due to different binding geometries between the coiled-coil and antiparallel helix structure and/or different monomer-dimer equilibria in the non-covalent forms.

The surface binding was also examined by varying the antigen density in a functional ELISA (Fig. 2), clearly showing a non-linear increase with coating density. This suggests that the avidity gain is indeed obtained when multivalent binding of the miniantibodies to the same surface becomes possible.

It is therefore possible to produce bivalent antibody fragments in functional form in E. coli. These miniantibodies may be the smallest bivalent structures still containing the complete antigen binding region. They also provide a direct route to the production of bifunctional antibodies in E. coli, which may become of interest in a number of medical applications.

We also investigated the use of the covalently linked antiparallel helix to make (Fab-helix)2 fragments in vivo (Kreber and Plückthun, unpublished). ELISA experiments showed that the avidity gain can be observed in a similar fashion, no matter whether the helix is linked to the light or heavy chain. In contrast, no (Fab')2 formation has been observed in vivo even at higher protein concentration in the periplasm of E. coli (Carter et al., 1992). Therefore, it is not the disulfide formation itself that causes the dimerization of the fragments. Rather, a non-covalent interaction of sufficient lifetime must first occur, which can merely be made permanent by a nearby S-S bond.

Folding in vivo

The antibodies are an ideal model system to investigate the problem of in vivo folding. While

The question of yield limiting steps in the functional heterologous expression of antibody fragments is still unanswered, investigations on a mouse Fab fragment (Skerra and Plückthun, 1991) strongly suggest that the periplasmic folding and/or assembly may be the limiting factor. Furthermore, the similarity in yields from Fv and scFv fragments is consistent with the idea that assembly is not a limiting process (Glockshuber et al., 1990). Different antibodies or those from different species may be limited at different levels (Carter et al., 1992).
It was therefore of interest to investigate whether folding catalysis in the periplasm may overcome this block. In a first approach, the periplasmic E. coli proline cis-trans-isomerase (Liu and Walsh, 1990) was overexpressed together with the antibody Fab, Fv, and both orientations of the scFv fragment (VH-linker-VL and VL-linker-VH) (Knappik, Walsh and Plückthun, unpublished data). As the Fab fragment, whose 3-D structure is known (Satow et al., 1986), contains 5 cis-proline peptide bonds, a folding block due to the slow isomerization of any of these bonds is conceivable. However, in all cases, the effects were rather small, suggesting that proline cis-trans isomerization is not the step, at which the folding pathway diverges from its desired path.

Additionally, the recently discovered protein disulfide isomerase from E. coli (Bardwell et al., 1991) was co-expressed with the Fab fragment, which contains a total of 5 disulfide bonds (Kreber, Beckwith and Plückthun, unpublished experiments). Again the effect was only marginal, although the functionality of the overexpressed isomerases could be clearly demonstrated. Similar results were obtained with human PDI (Pihlajaniemi et al., 1987) (Kreber, Skerra and Plückthun, unpublished data). This shows that the diversion of the folding pathway from the desired path does not occur at the step of disulfide formation or rearrangement, consistent with earlier mutant analysis (Skerra and Plückthun, 1991).

This approach of overexpressing potential folding modulators may be useful in pin-pointing bottle-necks during in vivo folding, and together with mutant analysis, perhaps lead to an increased understanding and handles for manipulating protein folding in the bacterial cell.

References


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