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Engineered antibody molecules

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Mono- and Bivalent Antibody Fragments Produced in *Escherichia coli:* Engineering, Folding and Antigen Binding

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INTRODUCTION

While the immune system can provide antibodies of high affinity and high selectivity to almost any antigen, their production requires animals and cell culture, and the overall structure of the antibody is fixed. The availability of monoclonal antibodies has created an explosion of knowledge about the antibody molecule, and with it a large number of different applications ranging from laboratory uses in almost all branches of biology, applications in biotechnology (e.g. protein purification), and chemical catalysis, to ambitious applications in tumor medicine. With these many uses, limits of the monoclonal technology become apparent. Small but highly stable fragments, antibodies of human origin, antibodies with extremely high affinities, fusions with enzymes, toxins and receptors or their ligands are now desirable. Furthermore, simplified methods for selecting and producing the antibody are needed.

The methodology of genetic engineering now makes it possible to devise pathways that yield tailor-made antibodies and do not depend on animal vehicles. Recent developments instrumental to progress in this field are distinctly multidisciplinary. The increasing number of crystal structures of antibody combining sites, especially complexes with antigens, now give a clearer picture of structural conservation and variability. Using this knowledge, smaller fragments can now be designed and linked in completely new ways. The technology to produce monoclonal antibodies has led to the determination of an enormous number of antibody sequences, mostly from the cloned DNA sequence. This knowledge was crucial for defining consensus sequences, which allowed rapid cloning of antibody genes by the polymerase chain reaction (PCR), thereby generating even more sequence information. Using recently developed bacterial expression technology,

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different types of fragments can now be made in fully functional form in *E. coli.* Thus, not only the effect of designed mutations but also the consequence of random changes can be rapidly investigated. This technology can be extended to clone and express libraries of chains from the immune system, to be screened for binding. An efficient screening methodology is derived from the surface expression on bacteriophages, which also exploits the correct periplasmic folding of the antibody during phage biosynthesis. This technology allows the enrichment of antigen-binding phages.

This account will summarize the basis of the developments in bacterial expression technology and the directly related problems of protein folding *in vivo.* Applications of this technology to the design of completely new types of molecules called "miniantibodies" will be discussed.

The immune system is a random mutagenesis and selection machine. Yet it has the experience of evolution built into the antibody design. To completely ignore the knowledge of structure and protein folding in random mutagenesis processes *in vitro* may not be the most efficient technological strategy for arriving at tailor-made antibodies. On the contrary, a clear delineation of the limits to variability set by the physical chemistry of protein expression and folding may allow a better focus on efficient mutational strategies.

Rational design and random mutagenesis strategies are thus not alternatives, but will cross-fertilize each other. From the structural analysis of designed mutations will come an understanding of crucial conformational switches, and from unexpected results of random mutants we expect to discover new interrelations in the protein.

ANTIBODY FRAGMENTS AND THEIR PROPERTIES

Fv fragments and their covalently linked derivatives

Antibodies are the archetypical multidomain proteins. From the 3D structures, it is now obvious that only the variable domains V_L and V_H make contact with the antigen. Thus, a fragment consisting of just these domains would be expected to contain the complete binding site, provided it were stable. This was first shown by proteolytic digestion of an antibody at about the same time as the first crystal structures became available (Inbar et al. 1972, Hochman et al. 1973, 1976, Givol 1991), and it showed the functionality of the Fv fragment in at least the investigated case. However, the proteolytic digestion does not easily lead to Fv fragments since the cleavage sites are not particularly preferred, except in special cases (Sharon & Givol 1976, Takahashi et al. 1991b). For instance, purification may become cumbersome, and the characterization difficult, if there is an excessive number of partially digested protein molecules (Givol 1991). As a case in point, a molecule assembled from a complete light chain and the V_H domain showed a

lower antigen-binding constant, even when the C_L domain was cleared off (Sen & Beychok 1986).

A more direct and general access to these Fv molecules was developed by using molecular biology, when it was shown that the Fv fragments could be functionally expressed in *E. coli* (Skerra & Plückthun 1988) (Fig. 1b, 2b) and in myeloma cells (Riechmann et al. 1988). A number of such unlinked recombinant Fv fragments has been reported since, produced by a variety of methods (see below; Ward et al. 1989, Field et al. 1990, Glockshuber et al. 1991, McManus & Riechmann 1991, Takahashi et al. 1991b, Cheadle et al. 1992), and full functionality seems to have been observed in the reported cases. However, one complication was noted (Glockshuber et al. 1990a): the affinity of the two domains[1)] V_H and V_L for each other is lower than the affinity of the light chain and the F_d fragment for each other (Bigelow et al. 1974, Hochman et al. 1976, Klein et al. 1979, Horne et al. 1982).

It may be part of the reason for the existence of C_H1 and C_L that they hold V_H and V_L together, thus putting fewer constraints on the variable domains to provide interactions to maintain the heterodimer structure. A range of stabilities of Fv fragments has been seen. For example, the Fv fragment of the lysozyme-binding antibody D1.3 has been called "stable" (although no dissociation constant was measured (Cumber et al. 1992)), and the Fv fragment of the phosphorylcholine-binding antibody McPC603 was investigated quantitatively and found to be of intermediate stability (V_L-V_H association constant 10^6 M^{-1}, Glockshuber et al. 1990a) and thus typical for the range of association constants reported (Horne et al. 1982 and references therein). It is possible that Fv fragments of low interaction energies between V_H and V_L, unable to stay associated during normal assays, may not have been reported in the literature. Part of the interacting surface is contributed by the hypervariable loops CDR3 of both chains and by the end of CDR1, and thus there is variability in the molecular interaction (Chothia et al. 1985). Therefore, a range of interaction energies is to be expected for different antibodies.

The consequence of this dissociation behavior of the Fv fragment can be an *apparent* loss of binding activity, which then showed a dependence on protein concentration. If this phenomenon is quantified, however, as has been done in the case of the antibody McPC603, it can be deconvoluted into a true hapten binding constant *identical* to the Fab fragment or the whole antibody, and the V_H-V_L dissociation constant measured independently (Glockshuber et al. 1990a). For instance, the antigen may stabilize the V_H-V_L complex, and this may lead to

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[1)] Abbreviations: V_H, variable domain of the heavy chain; V_L, variable domain of the light chain; C_L, constant domain of the light chain; C_H1, C_H2, C_H3, constant domains of the heavy chain; scFv fragment, single-chain Fv fragment; Fd fragment, heavy chain fragment consisting of V_H and C_H1.

nonlinear Scatchard plots. Thus, the true antigen dissociation constant is identifical for the Fv fragment and the whole antibody probably in most cases, and this is consistent with findings on crosslinked fragments (see below).

There are two principal ways to solve this V_H–V_L dissociation problem, if it occurs for the particular antibody of interest. One is to add the constant domains and work with Fab fragments. If, however, the small size of the Fv fragment is not to be sacrificed (as for applications in tumor biology or structural research), the two chains must be covalently linked.

Three ways have been investigated for linking the chains of an Fv fragment (Glockshuber et al. 1990a) (Fig. 1c–f). The first is chemical: crosslinking can be used, and in this case it was carried out with glutaraldehyde, the resulting imines being subsequently reduced with sodium borohydride. This method depends on the availability of surface lysines at appropriate distances between the two domains, but of course crosslinkers with different specificities for other amino acids can be used. To prevent modification of the antigen-binding site, this procedure can be carried out in presence of the antigen. The Fv fragments can then be used for binding measurements even at low concentrations (Glockshuber et al. 1991).

The second strategy is to use a natural crosslink: disulfide bonds (Glockshuber et al. 1990a). In the investigated prototype, the phosphorylcholine antibody McPC603 of known 3D structure (Satow et al. 1986) was investigated. The approach was purely geometrical. An algorithm was used (Pabo & Suchanek 1986) which identifies maximal overlap of the main-chain atoms of any pair of amino acids (one in V_H, the other in V_L) with the main chain atoms of any disulfide bond in the Protein Data Base. The approach was successful, and the two different disulfide bonds tested did form *in vivo* in *E. coli.* It has not yet been rigorously shown whether this approach will work routinely on antibodies with no known structure. However, framework positions can be identified in many cases where a substitution may be tolerated and such an approach might be successful, unless the antibody shows a severe departure from the range of typical V_H/V_L orientations and the particular geometries needed by the disulfide bond cannot be accommodated by shifts of the framework. It may be asked: why pursue this strategy at all? The main attraction of this approach lies in an enormous increase in stability provided against thermal denaturation by the intermolecular disulfide bond (Glockshuber et al. 1990a), much higher than any other crosslinking approach, including single-chain Fv fragments (see below).

The third strategy is to connect the two fragments by a genetically-encoded peptide linker to produce a so-called single-chain Fv fragment (Fig. 1d, e, 2c, d). This was first shown by Bird et al. (1988) and Huston et al. (1988), who produced inclusion bodies in *E. coli*, which could be refolded *in vitro.* Glockshuber et al. (1990a) then showed that this strategy is compatible with functional expression *in vivo.*

The fundamental problem is the spatial distance between the C-terminus of

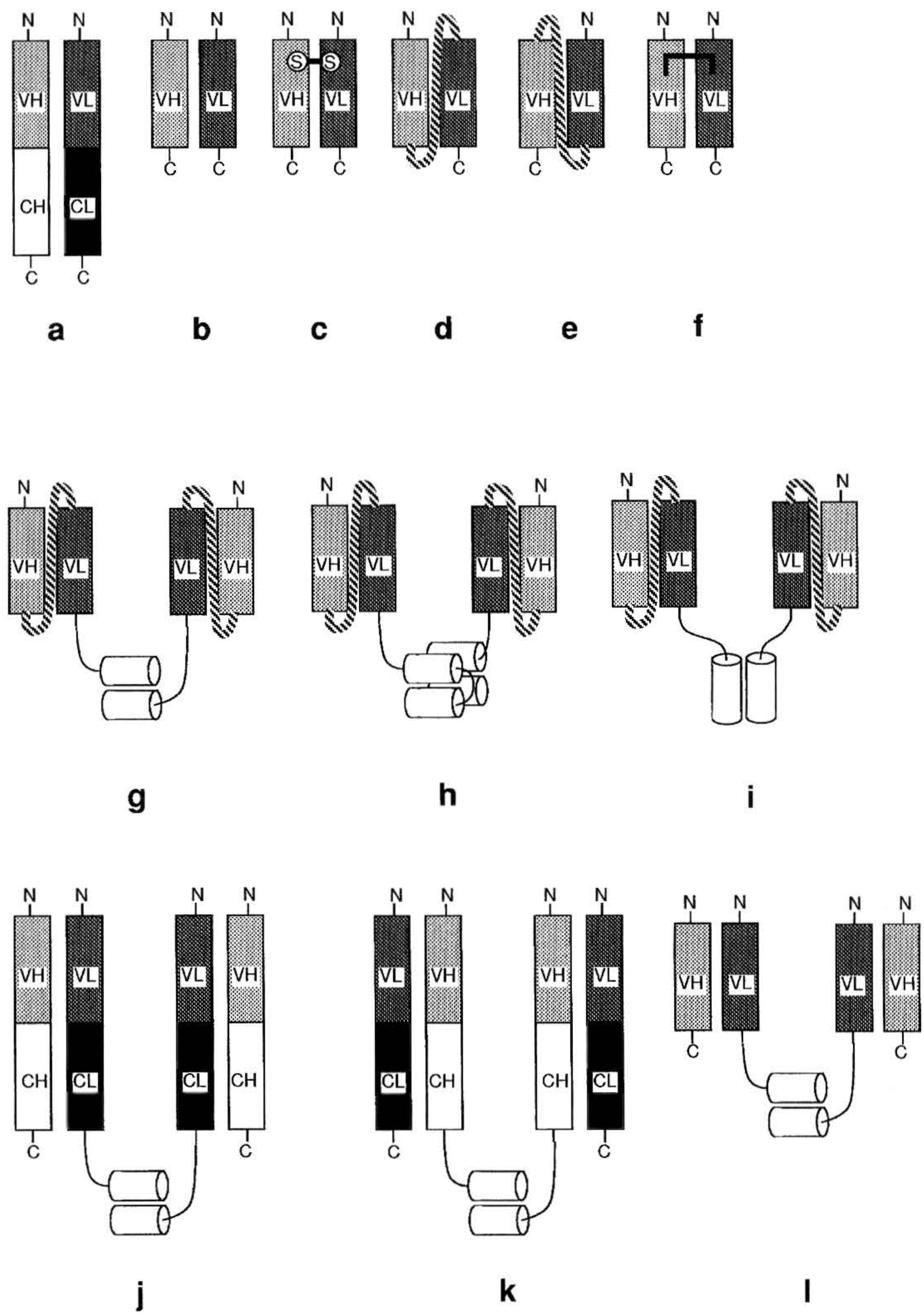

Figure 1. Antigen binding fragments of an antibody, functionally expressed in *Escherichia coli* to date. a) to f) describe functional monovalent fragments, g) to l) describe bivalent or bifunctional fragments. The constructs g) to i) have also been termed "miniantibodies". a) Fab fragment, b) unlinked Fv fragment, c) disulfide-linked Fv fragment, d) single-chain Fv fragment in the orientation V_H-linker-V_L, e) single-chain Fv fragment in the orientation V_H-linker-V_L, f) chemically crosslinked Fv fragment (crosslinked after periplasmic expression), g) miniantibody with one antiparallel bundle-helix on each single-chain Fv fragment, h) miniantibody with two antiparallel bundle-helices on each single-chain Fv fragment, i) miniantibody with one parallel coiled-coil helix on each single-chain Fv fragment, j) Fab fragments linked by antiparallel bundle-helix fused to light chain, k) Fab fragments linked by antiparallel bundle-helix fused to heavy chain, l) unlinked Fv fragments linked by antiparallel bundle-helix fused to light chain.

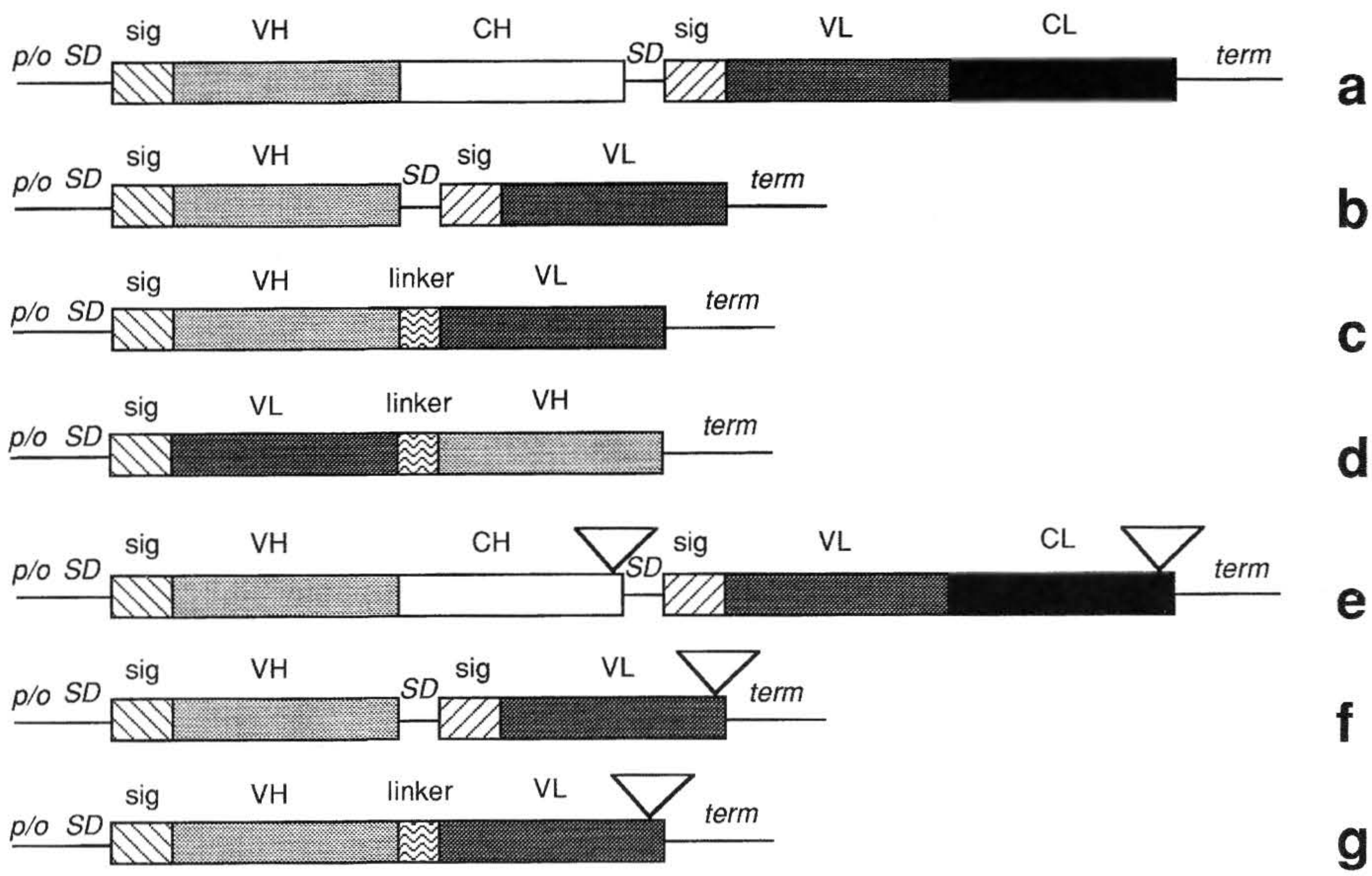

Figure 2. Arrangement of genes for functional *E. coli* expression of antibody fragments realized to date. a) Fab fragment, b) unlinked Fv fragment, c) single-chain Fv fragment in the orientation V_H-linker-V_L, d) single-chain Fv fragment in the orientation V_H-linker-V_L. e) to g) schematically describe the construction of the bivalent fragments, shown in Fig. 1g–l. The inverted triangle denotes an insertion of a synthetic cassette encoding the appropriate hinge and the helix and, if desired, the peptide extension covalently linking the two fragments. Using suitable restriction sites introduced at the ends of the genes, these cassettes can be combined with any fragment, and any cassette can be used with a particular fragment. In e) the insertion is at the end of the light or the heavy chain in the Fab fragment (corresponding to constructs in Fig. 1j and 1k). In f), the insertion is at the end of the light chain of the Fv fragment (corresponding to the construct in Fig. 1l). In g) the miniantibody arrangement is shown, which has been tested with several hinges and helices (corresponding to Fig. 1g to 1i).

one chain and the N-terminus of the other chain. This requires the linker to have a length of around 15 amino acids. A whole range of different linkers has been used (reviewed in Huston et al. 1991, Plückthun 1991b), and apparently there are no particular indispensable sequence requirements. Presumably, the linker is a passive entity during protein folding. The reported linkers are hydrophilic and flexible, and the sequence $(Gly_4Ser)_3$ has been used frequently. It is possible to exploit the lenient sequence requirements and introduce an affinity tag for detection purposes in this linker (Breitling et al. 1991). Both orientations of the single-chain Fv fragment, V_L-linker-V_H and V_H-linker-V_L, have been realized (summarized in Huston et al. 1991), in some cases even for the same antibody (see, e.g., Anand et al. 1991, Knappik et al. 1992). The free energy of folding was

very similar for both orientations in the case of the $(Gly_4Ser)_3$ linker in the antibody McPC603 (Knappik et al. 1992).

This suggests that the linker was indeed not contributing to the free energy of stabilization of the native state. This result is consistent with the length of the linker having only a slight influence on stability, above a minimum length, which would cause strain in the native state (Pantoliano et al. 1991). Another interesting observation from these studies is that only one equilibrium unfolding transition is seen for the scFv fragment (Pantoliano et al. 1991, Knappik et al. 1992); in other words, the scFv fragment behaves like a two-state system. This could mean either that the free energies of folding of the V_L and V_H domains are almost equal, requiring only a very small coupling energy between V_H and V_L to give a two-state folding behavior, or that the unfolding of the whole scFv fragment is a highly cooperative event, with no stable intermediate and a very large coupling energy (Brandts et al. 1989). We cannot yet rigorously distinguish between these possibilities, but we can note that the free energy of folding of the scFv fragment is higher by about 2 kcal/mol than that of the V_L domain for the antibody McPC603 (Knappik et al. 1992, Steipe et al., in preparation). The *in vivo* expression results of the two different scFv fragments of the antibody McPC603 (V_H-linker-V_L and V_L-linker-V_H), secreted into the periplasmic space of *E. coli* (see below) were also rather similar, in distinct contrast to observations of Anand et al. (1991). The reasons for this discrepancy remain unclear and may not be related to folding; in the latter case no *in vitro* folding data have yet been reported.

The three different covalent derivatives of the same Fv fragment, linked by glutaraldehyde, a disulfide bond or the linker peptide, were also compared in their irreversible thermal denaturation (Glockshuber et al. 1990a). All showed substantial stabilization compared to the unlinked Fv fragment, but they differed in the degree of stabilization. The most dramatic effect was achieved by the disulfide-linked fragments. These data suggest that the covalent linking of the two domains is an important factor, and perhaps the dissociation into V_H and V_L may be an early step in the denaturation pathway. However, the way in which the two domains are linked is of importance, too. The surface linkage via flexible lysine residues is of lower efficiency than the disulfide linkage, which links the framework much more tightly. The peptide linker has an even lower effect against irreversible denaturation, probably because the linker itself is somewhat flexible and disordered. For these reasons, the disulfide-linked Fv fragments may be an interesting class of molecules in cases where stability against denaturation is of importance.

The scFv fragments are an easy solution to the problem of linking both domains, not requiring intensive molecular modelling. Notably, the linker with the sequence $(Gly_4Ser)_3$ seems to be of general usefulness (Huston et al. 1991). ScFv fragments can be linked to affinity handles (Tai et al. 1990, Skerra et al. 1991, Lindner et al. 1992), reporter enzymes and toxins (Chaudhary et al. 1989,

Batra et al. 1990, Chaudhary et al. 1990a, b), at either the C-terminus or the N-terminus. However, an N-terminal location may interfere with the binding of the antigen, and the C-terminal location is the natural place for a fused domain.

For a few antibodies, dimers or even higher aggregates of the scFv fragments seem to be found (Mézes et al. 1992), which are not in rapid equilibrium. Most likely, they involve V_H–V_L pairing of two different molecules, but there is no detailed information about the arrangement of the domains. This process seems to be antibody-dependent and non-predictable, and it is not yet clear whether usually more than one binding site is functional or whether this may be exceptional. For critical applications requiring monomers, it may be useful to be aware of this phenomenon and size fragment the scFv preparation.

The Fv fragment and its covalent derivatives are the smallest fragments still containing the complete antigen-binding site. Two areas can be pinpointed where these fragments are of particular advantage. One is in structural research, as such small fragments generally give better ordered crystals than large Fab fragments (Bhat et al. 1990, Glockshuber et al. 1990b, Steipe et al. 1992). For NMR studies (Takahashi et al. 1991b, McManus & Riechmann 1991, Riechmann et al. 1992), fragments larger than the Fv fragment will currently not allow a complete assignment. The second area is that of tumor medicine. Tumor penetration is a well known problem with solid tumors, and scFv fragments show a much more even tumor penetration than whole antibodies or even Fab fragments (Yokota et al. 1992), although the total tumor localization is distinctly lower for scFv fragments, probably because of their rapid clearance (Colcher et al. 1990). However, bivalent "miniantibodies" (discussed below) have been constructed based on linked scFv fragments, and possibly these can be fine-tuned to achieve the desired clearance rate, retain bivalence and cause more efficient tumor localization but still be of a size small enough not to be restricted to the surrounding blood vessels. In applications where it is essential to restrict the protein surface to a minimum because of side reactions with other proteins or surfaces, Fv fragments may also be advantageous. Examples are immunoaffinity chorantography (Berry et al. 1991) and solid phase immunoassays.

In summary, Fv fragments and their linked derivatives have been shown to retain their antigen-binding affinity (Skerra & Plückthun 1988, Givol 1991), their 3D structure (Bhat et al. 1990) and, if the antibody is catalytic, their catalytic activity (Baldwin & Schultz 1989, Gibbs et al. 1991, Plückthun & Stadlmüller 1991). They contain the complete binding site, can be stabilized to similar or even higher degrees than the natural antibody and may be useful for many applications.

Single domains and single complementarity-determining loops

Is it possible to make the antigen-binding fragment smaller still? The next smaller entity is the single V_H or V_L domain. However, these are designed by nature to

coexist as V_H-V_L heterodimers in the context of an antibody. Both V_H and V_L have a rather hydrophobic interface, by which the heterodimer is stabilized (Chothia et al. 1985). For both V_H and V_L in the absence of the other, this seems to have different consequences. The hydrophobic nature of the V_H interface often leads to a limited solubility, and the idea of using V_H repertoires as a substitute for antibodies (Ward et al. 1989) must be examined with some scepticism. It is difficult to rule out the possibility that antigen binding does not occur non-specifically for V_H domains, at least in part at the very hydrophobic domain interface. The selectivity will probably be lower than that of whole antibody combining sites since in the latter case many nonspecific binding events would be eliminated by interference from at least one of the six loops. As a case in point, an Fv fragment as immunoaffinity ligand can separate two lysozyme species on a column, whereas the V_H domain from the same antibody does not (Berry & Davies 1992). Furthermore, at least some V_H domains will have poor solubility and thus be difficult to produce and thus perhaps even be absent from a library. While a solubility problem can probably be solved by engineering the interface, particular advantages of single V_H domains are not apparent. Because of combinatorial libraries (Huse et al. 1989) (see below), there is also no need anymore to restrict cloning to V_H domains.

V_L domains, on the other hand, may dimerize with themselves. The particular dissociation constant will vary from antibody to antibody because of the individuality of the contact residues involved, just as for the V_H–V_L association (Azuma et al. 1974, 1978, Maeda et al. 1976, Klein et al. 1979, Stevens et al. 1980). The association of V_L with V_L is structurally similar to V_H–V_L. If the V_L domain dimerizes, it will obstruct the binding site and will therefore not be suitable for binding the antigen. While the solubility of V_L often seems to be higher than that of V_H, the general disadvantage of lower binding energies and lower selectivity compared to whole antibody combining sites applies for the V_L domain as well, even if it does not dimerize.

V_L domains may, however, be interesting models for structural studies of antibody domains and binding sites. They can be conveniently prepared in *E. coli* either by chain separation from a secreted Fv fragment (Glockshuber et al. 1990b) or, more generally, by the secretion from *E. coli* and purification using immobilized metal affinity chromatography (Lindner et al. 1992). A high-resolution crystal structure of the V_L domain of McPC603, expressed in *E. coli* (Steipe et al. 1992), was compared to the same domain in the context of the Fab fragment, which was obtained by proteolysis of the mouse IgA (Segal et al. 1974, Satow et al. 1986). In these crystals, the V_L domains associate as dimers with very similar orientation of the two V_L domains as in the V_H-V_L heterodimer. No gross changes were found, consistent with the concept that the sequence of a domain alone determines its structure. However, some small but significant differences in CDR3 between the structure of the V_L domain and the structure of the same domain in

the context of the Fab fragment may be a consequence of interactions with the dimerization partner. Interestingly, in all three CDRs, conformations were found in this structure analysis (Steipe et al. 1992) that the canonical structure hypothesis (Chothia & Lesk 1987, Chothia et al. 1989) would not have predicted. This does not argue against the validity of this concept, but cautions about its predictive power. It may serve as a reminder of the very small database on which the predictions are currently based.

The particular crystal packing of the investigated V_L domain is commensurate with loop replacements in the CDRs, as they are not directly involved in crystal contacts. Thus, protein engineering may be used to study some of these questions of loop folding experimentally and thereby put future prediction efforts on a firmer basis by virtue of an increased database. In a first experiment, one CDR-1-grafted loop mutant was investigated (Steipe et al. 1992). This change was entirely local, with the rest of the structure being almost perfectly superimposable.

Of particular convenience is the fact that the recombinant V_L domains can be purified in a single step to homogeneity by immobilized metal ion chromatography, using a tail of five histidine residues (Lindner et al. 1992), just as had been demonstrated for the scFv fragment (Skerra et al. 1991). The protein can be crystallized immediately after dialyzing the column fractions and concentrating the protein, and the structure is indistinguishable from the V_L domain not having the histidine tail (Lindner et al. 1992). This tail appears disordered, but the structure was only determined in the absence of metal.

A V_L domain has also been studied by NMR (Constantine et al. 1992). The particular interest in studies of this type lies not so much in the deduced secondary structure, which holds few surprises, but in the information about the dynamics of this type of protein. Being apparently monomeric under the experimental conditions, thus V_L domain shows no readily discernible differences to the structure of the V_L domain in the Fab fragment, but gives evidence for slowly exchanging protons, which indicates stability of the structure, especially in the "middle" strands of the β-sheets.

Using still smaller entities as antibody surrogates will be difficult. Nevertheless, peptides derived from the antibody CDR region have shown binding activity to the antigen, although at affinity several orders of magnitude lower, even when they were constrained in conformations like the CDR (Williams et al. 1989, 1991, Saragovi et al. 1991, Taub & Greene 1992). Their use as an affinity ligand has not been successful (Berry & Davies 1992). Clearly, here lies a challenge for organic synthesis to create new and improved constrained molecules and supramolecular structures.

Fab fragments

Fab fragments are stable and have no problems of dissociation into the component chains. This stability is mainly due to non-covalent interactions, since it is also

seen with mouse IgA, which is not covalently linked, or with mildly reduced fragments (Bigelow et al. 1974, Klein et al. 1979, Alexandru et al. 1980). Fab fragments of certain antibody classes may be glycosylated in C_H1 (Nisonoff et al. 1975, Young et al. 1990, Skerra et al. 1990), but the most frequently used IgG molecules are not. This glycosylation which can occur, for example, in mouse IgA, does not have any effect on the binding of the antigen (Skerra et al. 1990), as might be expected from the 3D structure (Satow et al. 1986). The constant domains C_H1 and C_L can be useful in detecting the molecule with antibodies directed against them, and this has also been attempted by using V_H/V_LC_L molecules (Sawyer & Blattner 1991), although these molecules were not characterized further and the unpaired constant domain is a reason for questioning the stability and quaternary structure (Sen & Beychok 1986). However, much shorter tag sequences attached to the Fv fragment and its derivatives suffice for detection (Ward et al. 1989, Knappik et al., in preparation) and purification (Skerra et al. 1991, Lindner et al. 1992).

Using *E. coli* as an expression host, Fab fragments can be made in fully functional form (see, e.g., Better et al. 1988, Plückthun & Skerra 1989, Skerra et al. 1991, Carter et al. 1992) which are identical to the ones produced by proteolysis (Fig. 1a, 2a). Interestingly, the Fab fragment may give a lower functional expression than the Fv or scFv fragment of the same antibody. The reasons for this phenomenon are not completely understood (see below), but are linked to the periplasmic folding process. It is possible, however, that this difference can be masked or even be overcompensated if a particular Fv fragment or scFv fragment has an unusual sensitivity to *E. coli* proteases or an unusually low thermodynamic stability, and it may also be masked if the expression plasmid is poorly designed so that not even the periplasmic folding limit is reached.

Whole antibodies

A high level production of correctly assembled whole antibodies in *E. coli* has not yet been reported. The chains of whole antibodies have, however, been obtained in insoluble form from *E. coli* inclusion bodies (Boss et al. 1984, Cabilly et al. 1984). Most antibody species are glycosylated in C_H2, and others such as IgA and IgM can be glycosylated in C_H1 (i.e., in the Fab fragment) and other domains (Nisonoff et al. 1975). This glycosylation of C_H2 may be important for the assembly process and stability, since at least in IgG1, only sugar residues are involved in the interactions of the two C_H2 domains (Fig. 3c) (Sutton & Phillips 1983). Non-glycosylated IgG can nevertheless be produced in eukaryotic cells after treatment with the glycosylation inhibitor tunicamycin, or by genetically removing the glycosylation signal (Nose & Wigzell 1983, Leatherbarrow et al. 1985, Leatherbarrow & Dwek 1983, Duncan & Winter 1988, Walker et al. 1989, Tao & Morrison 1989). As deglycosylated IgG is devoid of biological functions

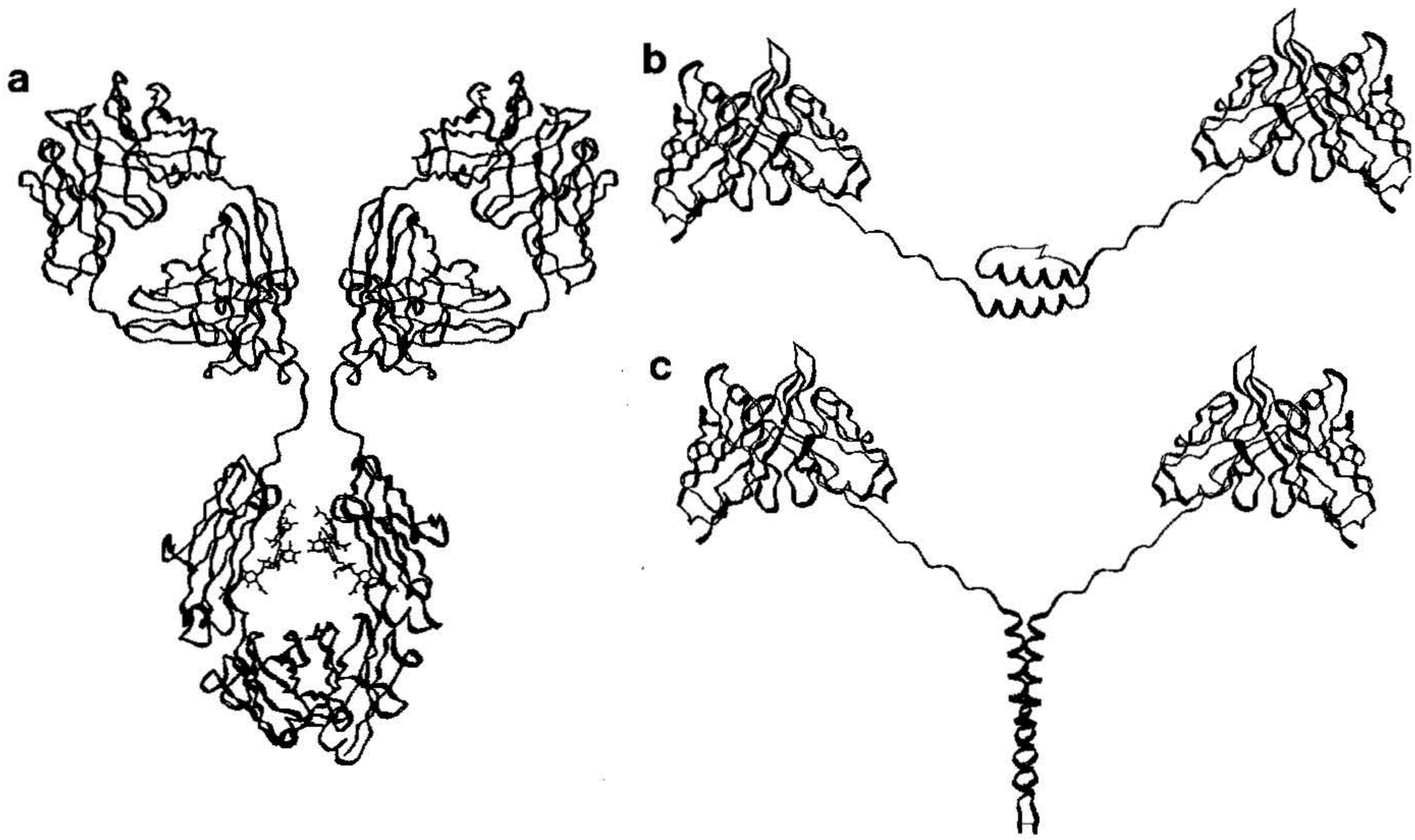

Figure 3. (a) Molecular model of the human antibody KOL. (b): Molecular model of the dimeric scHLXc miniantibody constructs derived from the single-chain F_v fragment of the mouse antibody McPC603 (Satow et al. 1986). The hinge region was modelled according to a polyproline-II helix with $\varphi = -78°$ and $\psi = 149°$. A standard α-helix with $\varphi = -57°$ 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. (c): Molecular model of the scZIPc miniantibody construct. The parameters are identical to those in A, except that a parallel coiled coil with about a quater turn of the superhelix was modeled for the leucine zipper part (O'Shea et al. 1991).

that depend on efficient binding to the Fc receptor and to complement, and deglycosylation has no dramatic effect on half life in serum (Wawrzynczak et al. 1992a, b), the production of non-glycosylated IgG would be mostly of academic interest. Most likely, the desired characteristics of such molecules, namely the absence of effector functions but the presence of bivalence, could be more easily met with $(Fab')_2$ fragments or the "miniantibodies" described below.

The glycosylation is also an intracellular sorting signal, and tunicamycin severely affects the secretion of IgA and IgE and, to a lesser extent, IgM, while IgG and IgD seem to be only slightly affected (Hickman et al. 1977, Tartakoff & Vassalli 1979, Vasilov & Ploegh 1982, Wall & Kuehl 1983, Leatherbarrow & Dwek 1983, Nose & Wigzell 1983, Leatherbarrow et al. 1985, Granato & Neeser 1987, Taylor & Wall 1988, Walker et al. 1989, Davis & Shulman 1989). Therefore, little is known about the properties of those unglycosylated antibody classes which cannot be produced in eukaryotic cells. It is possible that at least some of those would be severely disturbed in their folding and/or stability.

For whole antibodies, which would be of interest mainly because of their

effector function (summarized by Morrison (1992)), hosts other than *E. coli* may be more advantageous provided they lead to a glycosylation commensurate with function. Both mammalian cells (Morrison & Oi 1989, Shin & Morrison 1989, Page & Sydenham 1991, Bebbington et al. 1992) and other eukaryotes have been investigated as hosts (Horwitz et al. 1988, Hiatt et al. 1989, Haseman & Capra 1990, zu Putlitz et al. 1990) and it remains to be seen which of these methods will be most robust, convenient and productive in the long run on a laboratory scale and on a scale required for clinical trials.

It may be pointed out, however, that it can still be advantageous to carry out engineering and testing of the binding site or humanization with fragments expressed in *E. coli* (Carter et al. 1992) and to later transfer the final versions to a eukaryotic host for the production of a glycosylated molecule.

EXPRESSION IN *ESCHERICHIA COLI*

Many of the recent developments in the field of antibody engineering have profited from a methodology to rapidly produce and characterize genetically engineered antibodies. The method of producing antibody fragments in the native state in *E. coli* (Better et al. 1988, Skerra & Plückthun 1988) may be considered useful in this respect (Fig. 2). The *E. coli* production is rapid and thus allows the characterization of many variants in a relatively short time. It is also the basis for all screening for binding activity in libraries (see below). It may thus be useful to review the biochemical background of the expression and folding of antibody fragments in bacteria.

For any kind of screening which requires native and active molecules either to be present in the bacterial cell (i.e. the periplasm) or to be secreted from it, or displayed in correctly folded form on a phage, a process must be chosen which is compatible with the *in vivo* folding process. Even for mere production of a fragment (e.g. to characterize its properties in detail) native expression is attractive, because of the generality of the method. Refolding procedures will often have to be individually tailored to a particular molecule and the folded product will always have to be separated from misfolded forms for accurate quantitations. Furthermore, one-step purifications using antigen-affinity chromatography and process control using functional ELISA are arguments in favor of this methodology. Furthermore, in the analysis of mutants, the distinction between a failure to fold from a failure to bind can often be made, if the antibody protein isolated from the bacterial periplasm is analyzed for disulfide formation and chromatographic behavior.

However, there may be instances where other considerations may tip the balance in favor of refolding the antibody from inclusion bodies. If an antibody is to be labeled with isotopes on a large scale, and labor is of no concern, but the amount of culture broth is limiting because of the considerable expense of

the label (e.g. for NMR experiments), the refolding procedure may be attractive. Also, if one particular fragment of one particular antibody is to be produced routinely (e.g. for a large-scale clinical study), it is likely that refolding procedures with attractive yields can ultimately be worked out. Normally, however, it is the optimization of the *in vitro* folding and detailed analysis of the folded product, including a demonstration of full functionality and a homogenous folded state, which are the bottlenecks to producing an antibody by this procedure. It is not possible to extrapolate to future technical developments of expression methods accurately enough to forecast productivity, but native expression by periplasmic secretion has already given very attractive yields when combined with high cell density fermentation (Carter et al. 1992).

The various *E. coli* expression strategies have been discussed elsewhere in more detail (Plückthun 1991a, b). Secretion vectors have been described (Skerra et al. 1991) and remain under further development.

The question of how a native antibody fragment can be assembled *in vivo* is one of protein folding. The problem was first solved by the simultaneous secretion of both chains making up the antigen-binding site to the bacterial periplasm (Better et al. 1988, Skerra & Plückthun 1988), and it was shown that correct heterodimer assembly with full antigen-binding functionality was obtained, including correct processing of the signal sequence and disulfide formation. It appears that two factors may be required for the correct folding: first, the disulfide formation (see below), requiring the oxidizing milieu and the enzyme catalysis of the periplasm and, second, the concurrent folding of both chains, each perhaps acting as a folding template for the other.

The disulfide formation seems to be required for the stability of the variable domains (see below). The secretion of separate chains seems to lead to insoluble V_H and F_d fragments. Very probably, there will be some range of properties observed for various antibodies. The simultaneous folding requires that both chains are made in the same cell. The two most useful realizations of the requirement are the simultaneous secretion of both chains of the Fv or the Fab fragment using an artificial operon (Fig. 2) (Better et al. 1988, Skerra & Plückthun 1988), or the linkage of both domains V_H and V_L via a peptide linker to create one continuous protein, the scFv fragment, in which both domains are simultaneously secreted by necessity (Fig. 2c, d) (Glockshuber et al. 1990a).

It is possible to extend the expression strategy from defined antibodies to libraries in several ways. A particularly useful method is to randomly combine libraries of the heavy chain with those of the light chain, both obtained by PCR (discussed elsewhere in this volume) (Huse et al. 1989, Caton & Koprowski 1990, Persson et al. 1991, Duchosal et al. 1992). Each individual antibody molecule must, however, still be made by the *E. coli* cell in exactly the same way as described for defined molecules. One possibility to generate the combi-

natorial library is to introduce an expression vector, constructed according to the principles described in Better et al. (1988) and Skerra & Plückthun (1988), into the genome of a λ phage (Huse et al. 1989). The purpose of the phage is to provide a very good transfection efficiency – in a sense a biological equivalent of electroporation. This is crucial for obtaining a full representation of clones of the combinatorial library. The phage-infected bacteria then produce the antibody before or while they are lysing, and the product can be detected in the plaques by probing with radioactive antigen. While useful for making the library and screening it, in order to actually produce an antibody fragment the killing of the bacteria by the phage is to be avoided. Consequently, the expression vector is then excised from the phage and periplasmic secretion is carried out with just the plasmid as described above.

The location of the *in vivo* folding process in the periplasm has a number of interesting consequences. First, it easily lends itself to a secretion of the antibody fragment to the medium. This happens by itself even in the absence of any phage (Plückthun & Skerra 1989), and has been found to some degree with practically all *E. coli* strains and signal sequences tested (Schroeckh and Plückthun, unpublished), but the precise biological cause remains elusive. Apparently, the antibody protein itself causes some leakiness of the outer membrane, and this process is strongly dependent on growth and fermentation conditions. While this may be useful in very small-scale analytical work, on larger scales it is generally considered more practical to prepare the protein from the whole cell or the periplasmic fraction. At industrial production levels, breaking the cells may again be seen as more cumbersome.

The second consequence of the successful periplasmic folding is the compatibility of the antibody folding with display on the surface of a filamentous phage, such as M13 or fd. Since the N-termini of both the minor coat protein pIII (the product of gene III) and the major coat protein pVIII (product of gene VIII) are apparently exposed to the aqueous surrounding in the phage coat, extensions there are still compatible with phage assembly. This was first demonstrated for peptides displayed on the phage coat (Smith 1985, Parmley & Smith 1988, Scott & Smith 1990, Cwirla et al. 1990, Devlin et al. 1990, Greenwood et al. 1991, Felici et al. 1991). During phage assembly, these coat proteins are present as intermediates in the inner membrane of *E. coli* (reviewed in Russel 1991), with their N-termini exposed to the periplasmic space. Budding phage then coats its DNA with these proteins waiting in the membrane. Consequently, any protein folding of fusion proteins consisting of N-terminal antibody fragments and C-terminal phage coat proteins would take place in the periplasm, but anchored to the inner membrane via the C-terminal membrane domain. Therefore, the same kind of fragments which correctly assemble in the periplasm can also ultimately be displayed on filamentous phage, with important applications in affinity screening of libraries. Such experiments have been

carried out with Fab fragments (Kang et al. 1991, Chang et al. 1991, Hoogenboom et al. 1991, Garrard et al. 1991, Gram et al. 1992, Barbas et al. 1992) and single-chain Fv fragments (McCafferty et al. 1990, Barbas et al. 1991, Clackson et al. 1991, Breitling et al. 1991, Marks et al. 1991) and will be discussed elsewhere in this volume. Besides peptides and antibody fragments which can assemble in the periplasm, the surface display is possible with other proteins which fold in the periplasm of *E. coli* (Bass et al. 1990, Lowman et al. 1991, McCafferty et al. 1991, Roberts et al. 1992).

DISULFIDE BONDS

Disulfide bonds are so conserved in antibody domains that they have even been used as one of the criteria in the classification of distantly related molecules as belonging to the immunoglobulin superfamily (Williams & Barclay 1988, Hunkapillar & Hood 1989). The disulfide bond is usually completely buried within the hydrophobic interior of the antibody domains, and the accessibility to a probe of 1.4 Å radius is usually zero. In contrast, the intermolecular disulfides are not as inaccessible. This is true for both the natural ones linking C_L and C_H1, as well as for the engineered ones linking V_H and V_L.

Disulfide bonds form as a consequence of an oxidizing milieu and because of spatial proximity. However, the cytoplasm of all cells is thought to be reducing (Gilbert 1990), and the cell appears to have sufficient mechanisms to eliminate reactive oxygen species. One might speculate that the reducing environment of the cytoplasm is a remnant of early life in an anaerobic world, where all common machinery for DNA, RNA and protein synthesis and their precursors developed, and which incorporated mechanisms *requiring* free SH bonds, too difficult for evolution to redesign. From these considerations, cytoplasmic disulfide formation is certainly not excluded, but apparently it is not found for cytoplasmic proteins. In fact, the equilibrium value of formation of every disulfide bond is a composite value of (i) the chemical part (the intrinsic cysteine/cystine redox couple, a function of pH) and (ii) the protein contribution, i.e. the equilibrium of the two sulfurs being in productive contact vs. not being in productive contact. This latter factor would implicity contain all contributions from strain, steric clashes, solvation, etc. For the determination of whether disulfide formation occurs, however, there is another factor to be considered: the kinetic pathway of S-S bond formation and the question of its catalysis.

The native expression of correctly folded antibodies in reducing environments, e.g. by engineering disulfide-free variants of sufficient stability, may be a general way to achieve an "antisense protein", which may be used to titrate out certain factors within the living cell. Interestingly, however, there have been reports suggestive of functional antibodies from the cytoplasm of yeast and myelomas without any change to the antibody protein (see below).

To find answers to these problems, the question was first asked as to whether antibody fragments actually need disulfide bonds for stability. This problem was studied using a number of mutants and fragments of one defined antibody, the anti-phosphorylcholine mouse IgA McPC603. In examining the resistance of the folded Fv fragment to mercaptoethanol, it was found that the exposure leads to a time-dependent precipitation. The reaction is of first order and the rate is proportional to the thiol concentration. In measuring the temperature dependence of the reaction, a transition temperature was found (Glockshuber et al. 1992). Taken together, this suggests a model in which the domain first to has "melt" to allow the rate-limiting attack of the thiol at the cysteine sulfur, and then a fast precipitation of the protein occurs. Apparently, the reduced Fv fragment is not stable.

Consistent with this idea, a refolding of the reduced and urea-denaturated Fv fragment was not possible without oxidation (Hochman et al. 1976, Glockshuber et al. 1992). Quantitative *in vitro* folding experiments have been carried out with single-chain Fv fragments (Huston et al. 1991, Freund et al., unpublished), a single-chain immunotoxin (Buchner et al. 1992), Fab fragments (Haber 1964, Rowe 1976, Buchner & Rudolph 1991), and isolated domains (Rowe & Tanford 1973, Goto & Hamaguchi 1982, Goto et al. 1988), but no *in vitro* refolding of variable domains in the absence of S-S formation has been reported, although simple air oxidation was occasionally used for Fv fragments (Hochman et al. 1976, Glockshuber et al. 1992). Goto & Hamaguchi (1979, 1986) reported that a C_L domain appears to assume a native-like structure of lower stability, if the intradomain disulfide bond is reduced. In contrast, *in vitro* folding of antibody domains containing the correct disulfide bond is often possible with high yields (Pantoliano et al. 1991, Glockshuber et al. 1992). In these experiments, the native protein was first denatured and then renatured, without ever reducing the protein.

To correlate these findings with *in vivo* folding in bacteria, the disulfide bonds of the variable domain were removed, by replacing the cysteines with alanines, then with other branched hydrophobic amino acids in various combinations, and finally by replacing each of these cysteines, one by one, with tyrosine (Glockshuber et al. 1992). The rationale for this last choice, seemingly unsatisfactory for structural reasons, was that it has been observed in nature: In the antibody ABPC48, such a substitution has been found at Cys H98 (consecutive numbering), and the functionality of this antibody has been demonstrated (Rudikoff & Pumphrey 1986, Victor-Kobrin et al. 1990). Each of the four cysteines was individually replaced by tyrosine, and the above exchanges were then carried out in the context of the Fv fragment, the scFv fragment and the Fab fragment, all expressed in the periplasm of *E. coli*. The result was that none of the substitutes led to a functional antibody fragment, as determined by hapten affinity chromatography, a method with which all three w.t. fragments can be easily purified. Instead, processed but only partially soluble fragments were obtained and detected

by Western blot, presumably because they precipitated after transport. It must be concluded, therefore, that none of the antibody fragments is able to fold properly in the absence of any one of the disulfide bonds in the variable domains. Taking into account the limited stability of antibody domains even in the presence of the S-S bond (see above), these results taken together appear to be consistent with the idea that the reason for the lack of folding is a thermodynamic one: the folded state is of insufficient free energy without the disulfide bond.

In a further set of experiments, the cytoplasmic expression of the Fv, the scFv and the Fab fragment of the antibody McPC603 was investigated. In no case was a functional fragment observed, as defined by material able to bind on a hapten affinity column, although all three proteins were made as soluble material (Schmidt and Plückthun, unpublished). Taken together, these results are all consistent with the notion that at least the antibody McPC603 cannot reach an antigen-binding state in the absence of disulfide formation in the variable domains, and in the cytoplasm of *E. coli* disulfide formation does not take place under these conditions. If disulfide formation is prevented by chemical reduction, by mutation, or by expression in a reducing environment, no functional protein is observed.

What is the reason for the difference between McPC603, by all accounts typical, and the antibody ABPC48, containing a CysH98-Tyr mutation apparently still giving sufficient stability? It is possible that a relatively constant loss of free energy is suffered by all fragments from which a disulfide bond has been amputated. Those having a higher stability to begin with would then still be able to reach the native state. As there is a range of stabilities of antibody domains, there would be a fraction of antibodies still able to fold without a disulfide, while others would not. It is currently completely unclear which proportions of antibodies belong to either category.

Since neither the refolding of the antibody ABPC48 *in vitro* nor its behavior as Fv, scFv or Fab fragments in *E. coli* has yet been studied, it remains a formal possibility that there exists a folding pathway in the higher cell that is not dependent on an early disulfide formation, leading to the final folding state perhaps only accessible via a particular molecular chaperone. Thus, we cannot yet rigorously rule out the possibility that the folding problems of the disulfide-free antibody fragments tested are of a kinetic nature, and not a thermodynamic one.

Unexpectedly, there have been some suggestive reports above the functional expression of antibodies and antibody fragments in the cytoplasm of eukaryotes (Biocca et al. 1990, Carlson 1988, Tang et al. 1991). From these experiments, and one report about such an observation in *E. coli* (Cabilly 1989), it is quite difficult to rigorously exclude folding and disulfide formation after opening the cells (Wood et al. 1985). Furthermore, these antibodies have not been characterized in detail to determine the extent of any disulfide formation. Yet the report about

an anti-alcohol dehydrogenase (ADH) antibody expressed in the cytoplasm of yeast, suggested to show a very slight ADH inhibition (Carlson 1988), and the observation that an antibody with chorismate mutase activity can suppress the defect of the cytoplasmic enzyme encoded by the host chromosome, albeit only in the context of another unspecified chromosomal mutation (Tang et al. 1991, Bowdish et al. 1991), invites speculation that at least some disulfide formation might occur under these circumstances already in the cytoplasm of yeast, and perhaps higher cells (Biocca et al. 1990).

The exact pathway of folding of antibody variable domains has remained unclear, as it is for most other proteins. In particular, the temporal order of the collapse of the chain, formation of secondary and tertiary structure and disulfide formation is not known. It must be kept in mind that the disulfides are inaccessible in the final structure and any sulfhydryl oxidizing reagent involved in their formation must act before then. Hochman et al. (1976) have proposed that disulfide formation must be allowed to occur in a largely unfolded state. This leads to a peculiar *in vitro* folding procedure in which an oxidation is required before the denaturant is removed. This procedure does not always maximize the refolding success, however (Huston et al. 1991, Freund et al., unpublished observations), even when air oxidation is replaced by the chemically more defined thiol/disulfide interchange under various conditions, suggesting that different pathways may be preferred by different antibodies, and that parallel pathways may be of very similar activation energies such that only small changes in the antibody structure are necessary to favor one pathway over another.

PROTEIN FOLDING *IN VITRO* AND *IN VIVO* (*E. COLI*)

The process of folding of a secreted protein in *E. coli* must follow many steps. Certainly, complicated molecules, such as antibody fragments consisting of several domains, even separate chains, disulfide bonds and *cis*-peptide bonds will have a complicated folding pathway.

After the synthesis of the precursor on the ribosomes in the cytoplasm, the protein must be prevented from folding, since the actual crossing of the membrane will occur in a non-native state (Eilers & Schatz 1986, Randall & Hardy 1986)). The signal sequence itself, while not preventing folding, appears to slow it down. Furthermore, since the folded state seems to be unstable in the absence of disulfide bonds, the folding of the precursor protein is probably not an important side reaction in the case of antibody chains in *E. coli. E. coli* has at least two cytoplasmic proteins which can interact with precursor proteins, SecB and GroE (Kumamoto 1991, Laminet et al. 1990). Their specificity remains unknown, and while an scFv fragment does interact *in vitro* with GroE (Zahn & Plückthun 1992), it is unclear whether its precursor interacts *in vivo*. A side reaction on the way from the ribosome to the membrane might possibly exist in the form of

proteolysis of the precursor, but the quantitative importance and the sequence specificity of such hypothetical proteolysis remain to be investigated. An aggregation of the precursor protein was observed for β-lactamase only at high temperature (Laminet & Plückthun 1989) and, in general, precursor proteins do not accumulate.

After traversing the membrane, the actual folding must occur. Two types of chemical steps have been identified as slow in protein folding (Fischer & Schmid 1990): The isomerization of *cis*-peptide bonds N-terminal to proline (proline *cis-trans* isomerization) and the formation and possible rearrangement of disulfide bonds (Freedman et al. 1989). In addition, however, other non-covalent steps in the folding process can be slow and rate-limiting as well, such as readjustment in the formation of the packed hydrophobic core (Jaenicke 1991).

One of the experimental facts observed in the study of periplasmic folding of antibody fragments was that a smaller percentage of Fab fragments reaches the correctly assembled native state than of the smaller Fv fragments, despite the fact that both are produced in the same expression strain and vector and differ only by the presence of the constant domains (Skerra & Plückthun 1991). The *total* amount of both proteins appears to be more similar, if the material detectable by Western blot in soluble and insoluble fractions is considered together. Furthermore, only a trace of precursor protein is detectable under these conditions, suggesting that the transport through the membrane is not limiting the assembly process, but only at still higher expression levels. The use of a stronger promoter simply increases the amount of insoluble material under these conditions. In combination, these data suggest that it is the periplasmic folding process which is not proceeding with the same efficiency for the Fab fragment as it does for the Fv fragment.

It was therefore of interest to investigate the reasons of this difference. The first possibility was the higher number of disulfide bonds of the Fab fragment, which has five, compared to the two in the Fv fragment. The mouse IgA Fab originally investigated (having four typical intradomain disulfides, plus an untypical one in C_H1, plus an unpaired cysteine at the end of C_L) was compared to three variants: one in which the "untypical" disulfide in C_H1 of IgA was removed, one in which the free cysteine of C_L was removed, and one in which an IgG1 domain was substituted, thus forming an H-L disulfide bond. In all cases, completely functional Fab fragments were obtained from *E. coli* in similar amounts of correctly folded protein as was obtained with the original fragment (Skerra & Plückthun 1991).

The same question was investigated by over-expression of the *E. coli* disulfide-isomerase (Bardwell et al. 1991, Kamitani et al. 1992), a periplasmic enzyme, together with the antibodies. While the over-expression did lead to the expected substantial increase in periplasmic enzymatic activity of the isomerase, it did not influence the partitioning of the Fv fragment, the Fab fragment or the scFv fragment between the native and the non-native state (Knappik et al. 1992). A

similar experiment with the human disulfide isomerase did not influence the proportion of correctly folded Fab fragment either. However, the *E. coli* disulfide isomerase *is* essential, since in a strain not producing this enzyme, almost no functional antibody is produced (Knappik et al. 1992).

The second process, which is often a slow step in protein folding, is the *cis-trans* isomerization of peptide bonds N-terminal to proline. There are five *cis*-prolines in the structure of the Fab fragment of McPC603 (L8 and L101 in V_L, L147 in C_L and H143 and H155 in C_H1, [residues given in consecutive numbering]; Segal et al. 1974, Satow et al. 1986). Only two of them are in the Fv fragment. Again, it seemed reasonable to ask whether the increased number of *cis*-peptide bonds might be responsible for a lower fraction of the Fab fragment assuming the native structure. Originally proposed by Brandts et al. (1975), it is now generally assumed that peptide bonds are formed in *trans* and that the *cis*-configuration must be obtained by isomerization during protein folding (summarized in Stewart et al. 1990). In a first experiment, one loop in the C_H1 domain of the Fab fragment was completely exchanged (Skerra & Plückthun 1991). This loop is at the opposite end of the molecule from the antigen-binding site. In the original structure two *trans* prolines and one *cis*-proline are present. In the replacement loop, which was taken from the C_H1 domain of human IgA, only one proline is present, which is very probably in *trans*-configuration. This loop replacement leads to a completely functional antibody, albeit at similar partitioning ratios of the folded to unfolded protein.

Again, this question was also investigated by the over-expression of a suitable catalyst. An enzymatic function was discovered (Fischer et al. 1984), apparently ubiquitous, which at least *in vitro* accelerates the isomerization of the peptide bond (Fischer & Schmid 1990). *E. coli* possesses two such enzymes, one cytoplasmic, the other periplasmic (Liu & Walsh 1990, Hayano et al. 1991). Since the crucial process appears to be the periplasmic folding, this latter enzyme was over-expressed. The increase in activity could be demonstrated, but the effect on the yield of correctly folded Fab fragment was negligible (Knappik et al. 1992).

The conclusion from these experiments is that apparently the isomerization of *cis*-peptide bonds and the formation or rearrangement of disulfide bonds is not what limits the periplasmic folding process. This is quite remarkable, as there are examples of the sensitivity of the *in vitro* folding of antibody fragments to catalyis of exactly these steps (Gato & Hamaguchi 1982, Lang & Schmid 1988, Buchner et al. 1992). It thus appears as if another process, which may mask the action of these putative folding catalysts, such as the aggregation of folding intermediates, limits the assembly process in the periplasm of *E. coli.* An investigation of suitable molecular chaperones perhaps able to divert aggregation is now being conducted. Interestingly, the primary sequence of the Fab fragment seems to determine the size of the folding bottleneck (Carter et al. 1992), i.e. the magnitude of the flux to unwanted off-pathway reactions.

Yet, folding bottlenecks are not limited to Fab fragments. At higher temperature insoluble protein has been observed with scFv fragments (Colcher et al. 1990, Gibbs et al. 1991, Knappik et al., unpublished) despite their expression with a signal sequence. Very probably, the protein is transported and precipitates in the periplasm, since the signal sequence seems to be removed. The precipitated scFv can be refolded *in vitro*. Similar results have been obtained with a V_H domain (Power et al. 1992), although in this case not only a kinetic folding bottleneck but also the poor solubility of the final product may present a problem (see above), and no folding was reported. High temperature is not suitable for transport in *E. coli* and, under these conditions, periplasmic inclusion bodies have been observed for other proteins (Libby et al. 1987, Wong et al. 1988, Bowden et al. 1991). It is likely that this happens for the antibody proteins as well under these conditions. Nevertheless, the environmental factors can be taken care of by using a lower growth temperature leading to increased folding *in vivo* (Glockshuber et al. 1990a, Skerra et al. 1991, Skerra & Plückthun 1991) just as in other proteins (Takagi et al. 1988). The remaining folding bottlenecks are a function of the protein itself. However, it is possible that these factors can be identified and alleviated by future research.

RECOMBINANT BIVALENT ANTIBODIES ASSEMBLING IN *E. COLI*

Probably the most efficient way to increase the equilibrium constant of an antibody to a surface (a cell surface or the surface in an ELISA well) is to make use of the multivalency effect. This is a general physical principle and thus, at first approximation, applies independently of the nature of the antigen and of the binding site. It probably constitutes the most reliable and efficient way to increase effective binding constants to a surface given the present stage of knowledge, as it is dependent only on the geometric constraint of binding two antigen molecules simultaneously, and it can still be combined with affinity-enhancing changes in the binding site.

Perhaps the most straightforward concept to quantify this phenomenon is that developed by Crothers & Metzger (1972). It says that the apparent binding constant of a bivalent antibody, K_{obs}, usually called avidity, can be thought of[2)] as the product of two binding constants $K_{obs} = K_1 \cdot K_2$. The affinity constant $K_1 =$

[2)] This abbreviated derivation has been simplified to the point of only comparing the binding of a monovalent fragment to the same surface as the bivalent fragment. Also, the problem of dimensions has been treated pragmatically: Concentrations have their usual dimensions (i.e. in mol/L), the affinity constant K_1 and K_{obs} are inverse concentrations (L/mol), K_2 must be dimensionless, and the units of area and length must be given in dm and dm^2 to be commensurate with L ($= dm^3$). For a rigorous derivation, a discussion of standard states and the comparison with binding to a monomeric antigen, See Crothers & Metzger (1972).

$[Ab \cdot Ag]/[Ab] \cdot [Ag]$ is the second order constant seen with monomeric fragments to a surface of the same type, where [Ab] is the free antibody concentration, [Ag] is the free antigen concentration, and $[Ab \cdot Ag]$ the concentration of complex. K_2 is a first order constant, which is equal to K_1 when the effective concentration of the second antigen binding site, $[Ab]_{eff}$, is substituted which applies when the first one is bound to its target epitope. This binding site concentration $[Ab]_{eff}$ is equal to 1 molecule in a hemisphere centered at the first binding site, which has the radius r of the average distance between the two binding sites. This needs to be converted to molar units by dividing by Avogadro's number N_{Avo}:

$$[Ab]_{eff} = \frac{1}{\frac{1}{2} \cdot \frac{4}{3} \cdot \pi \cdot r^3} \cdot \frac{1}{N_{Avo}}$$

The surface concentration x (epitopes per unit area) of the antigen determines how many molecules the second binding site can reach once the first is bound, namely those present in a circle with radius r $x \cdot \pi \cdot r^2$ (ignoring complications from already occupied binding sites). This is the number of epitopes within the circle which the second site can span after binding of the first, and it thus gives a statistical weight to the binding probability. After multiplying by the statistical factor of 2 for the two equivalent binding sites, this leads to the following formula:

$$K_{obs} = 2 \cdot K_1 \cdot K_1 \cdot \frac{1}{\frac{1}{2} \cdot \frac{4}{3} \cdot \pi \cdot r^3} \cdot \frac{1}{N_{Avo}} \cdot x \cdot \pi \cdot r^2$$

or

$$\frac{K_{obs}}{K_1} = \frac{3}{N_{Avo}} \cdot x \cdot \frac{1}{r} \cdot K_1$$

This (simplified[2]) equation of Crothers & Metzger expresses the intuitively appealing relation that the gain by the multivalence effect K_{obs}/K_1 should be proportional to the binding constant of the monovalent fragment K_1. Also, the effect is of course only seen if there are epitopes in reach of the second arm, and it is proportional to their number x. This is probably the most important consideration. Epitopes of very large proteins may not be able to come together close enough for the antibody to reach the neighboring site. On the other hand, they may be close enough but have a relative orientation towards each other such that a simultaneous binding of both epitopes by the same bivalent antibody is not possible. Clearly, the same laws apply if two *different* epitopes are used to obtain the multivalence effect with a bispecific antibody, recognizing one epitope with each of its arm, and this may be an alternative for achieving tight binding to a cell surface if the first approach does not lead to success.

From these considerations, it may appear to be useful to make the arms as

long as possible. However, the local concentration of the second binding site is inversely proportional to this length, r, and thus the avidity gain becomes very small. Furthermore, practical engineering problems impose a size limit on this length.

Clearly, many complications are neglected in this model, such as inadequacies describing surface binding with terms from solution chemistry, the energy required to "bend" the antibody or the antigen, medium changes close to the surface, other steric penalties, or the saturation of the surface occurring at higher antibody concentration, which competes with divalent binding. Thus, quantitative estimates should not be taken too far, especially since there is uncertainty in each of the variables. However, it is important to clarify the proportionalities within this relationship.

To approach these problems with fragments producing dimers in bacteria, the use of "dimerization handles" was investigated (Pack & Plückthun 1992). Briefly, single-chain Fv fragments were fused via a flexible hinge-region to several kinds of amphipathic helices (Fig. 2g-i, Fig. 3), which act as a dimerization device. The resulting "miniantibodies" are dimeric, fold and dimerize *in vivo* in *E. coli* and do show the expected gain in avidity. Amphiphilic helices seemed the most interesting candidates as dimerization handles, since they are small and much is known about their structure and physical properties. An important consideration was the compatibility of the helices with transport through the membrane as this seems to be a requirement for the correct folding and disulfide formation in bacteria (see above).

Two basic molecular structures of associated helices have been studied in more detail: An antiparallel four-helix bundle or a parallel-coiled coil (Fig. 3).

The design of four-helix bundles has been described (Eisenberg et al. 1986, Ho & DeGrado 1987, Regan & DeGrado 1988, Hill et al. 1990) but only circumstantial evidence exists for the precise molecular association. A crystal structure has been seen for a shortened version of the helix-forming peptide (Eisenberg et al. 1986) but a direct demonstration of this arrangement for this sequence is still lacking, and structures other than a four-helix bundle have been considered. Nevertheless, there is precedence for this arrangement in natural proteins.

The four-helix bundle can in principle be made up from four single helices, or two-helix dimers, or one continuous four-helix chain. For obtaining dimers, and perhaps tetramers, the first two possibilities were investigated (Fig. 1g, h, 3b). To allow flexibility of the antibody construct, which is probably necessary for adapting to a surface containing the antigen, a hinge region was introduced between the scFv fragment and the helix. In the initial design, the upper hinge of mouse IgG3 was used. In experiments comparing the flexibility of various hinge regions (Dangl et al. 1988, Tan et al. 1990, Burton 1990) it was noted that this hinge led to a high degree of flexibility of the two antigen-binding arms with respect to each other. The upper hinge of IgG3 is fairly long, and while it was

not the most flexible one in these studies, being slightly less so than IgG2 and IgG2b, it was chosen because of its hydrophilic nature. Indeed, this hinge is commensurate with membrane transport in *E. coli.* The length of the hinge would permit the two scFv fragments to have about the same orientation in space as the two binding sites of a whole antibody and to adapt to the orientation of surface-bound antigen.

This IgG3 upper hinge itself, despite its length and its probably extended molecular structure, is surprisingly resistant to bacterial proteases. In analogy to a peptide model studies by NMR (Kessler et al. 1991) and some electron density seen in the crystal structure of the KOL antibody (Marquart et al. 1980), the hinge structure is probably similar to a polyproline-II helix. Interestingly, the nature of the dimerization domains seems to determine the extent of proteolysis at the end of the same hinge. At the present time, there are no data available about the resistance of these hinges to serum proteases.

More recently, a number of different natural and synthetic hinges have been tested (Pack and Plückthun, in preparation), which may be useful when flexibility is less of a problem than proteolysis. The modular design of the system, however, allows the combination of any hinge with any dimerization domain.

The sequence of the four-helix bundle peptide was taken from the work of Eisenberg et al. (1986). In this first construct, only one helix was fused to the hinge. Despite the design, however, no evidence for major tetramer formation was obtained. Ultracentrifugation experiments were instead consistent with a monomer-dimer equilibrium (Pack & Plückthun 1992). This is in line with the relatively weak equilibrium association constant of $4.2 \cdot 10^{13}$ M^{-3} for the monomer/tetramer equilibrium as measured for the isolated peptides (Ho & DeGrado 1987).

To stabilize the dimers against dissociation, a hydrophilic peptide tail was added, which carried a cysteine residue at the end (Fig. 3). Indeed, covalently associated dimers were obtained (Pack & Plückthun 1992), suggesting that the lifetime of the helix is long enough to allow dimer formation in the periplasm of *E. coli.* This suggests that disulfide formation is a consequence of dimerization and not a cause. Consistent with this idea, Carter et al. (1992) observed that even at a very high concentration of a periplasmic $F_{ab'}$ fragment, no $(Fab)'_2$ fragments form. This only happens if a piece of the hinge is added which also dimerizes as the peptide alone (Moroder et al. 1990), similar to the situation with the helices.

Another way of improving the stability of the four-helix bundle was to fuse two helices in tandem, separated by a turn-encoding peptide, onto one fragment (Pack and Plückthun, in preparation). Thus, the four-helix bundle is directly created from two parts, as e.g. in the natural protein Rop (Banner et al. 1987). This also leads to very stable dimeric miniantibodies.

In a second design, the ability of certain sequences to form coiled-coils was exploited (Fig. 1i, 3c). This dimerization device is used by nature in transcription

factors (Landschulz et al. 1988), but has also been fused to other proteins (Hu et al. 1990, Blondel & Bedouelle 1991). In the original design (Pack & Plückthun 1992), the helix from the yeast transcription factor GCN4 was used, the crystal structure of which has been determined (O'Shea et al. 1991). Again, the helix was either used as such or it was extended by a short peptide ending in a cysteine residue. Since the helices are now parallel, the cysteine-carrying peptide can be much shorter. With the cysteine-containing zipper, covalent association was observed, while ultracentrifugation studies were consistent with both the non-covalent and the covalent versions forming dimers (Pack & Plückthun 1992).

The crucial question was of course whether any of these miniantibodies showed the expected gain in avidity, compared to the monomeric fragments. This is the case, and the miniantibody containing the antiparallel covalent helix dimer comes very close to the whole antibody as determined by its avidity. There is some difference between the surface binding of the various constructs, and at present we can not rigorously distinguish geometric effects (constraining the orientation of the two scFv fragments more in some constructs than in others) from stability effects during the assay. However, the binding to the surface was shown to be functional by inhibition with soluble antigen and dependent on the surface concentration of the antigen (Pack & Plückthun 1992, Pack et al. 1992).

The miniantibodies are thus compatible with *in vivo* folding in *E. coli* and must assemble to dimers already in the cell, since they can be isolated as such by antigen affinity chromatography. Even the formation of the disulfide bond linking the helices, in those cases where cysteines are present, seems to occur spontaneously. The linking of Fv fragments and Fab fragments by the same methodology was also investigated (Pack, Krebber and Plückthun, unpublished). However, complications with chain dissociation in the Fv fragment of McPC603 and some lower expression levels in the case of Fab fragments make these fragments less appealing.

Besides giving a gain in avidity, this design opens a new access to bispecific antibodies using the *E. coli* technology. Previously, bispecific antibodies have been prepared by chemical crosslinking of whole antibodies (Staerz et al. 1985, Perez et al. 1985) or monovalent fragments (Nisonoff & Mandy 1962, Raso & Griffin 1981, Brennan et al. 1985) as well as by the hetero-hybridoma technique (Milstein & Cuello 1983, Suresh et al. 1986, Takahashi et al. 1991a) and transfection of hybridoma cells with an expression plasmid for the second antibody (Lenz & Weidle 1990). All co-expression approaches with two different specifities, in which the H and L chains for a particular binding site are not linked, suffer from the problem of random H-L association, and random (HL)-(HL) association, leading to only a small percentage of correct product. This problem is observed in the hetero-hybridoma approach as it is in co-transfection experiments and leads to difficult purification schemes.

There has been much interest in the field of tumor immunology in using such

principles to (i) obtain a greater selectivity for a tumor cell by combining more than one specific marker and (ii) to recruit T cells for cell killing by combining an anti T-cell specifity with a tumor specifity (overviews: Staerz & Bevan 1986, Jung & Müller-Eberhard 1988, Sedlacek et al. 1988, Waldmann 1991, Nelson 1991, Fanger & Guyre 1991). However, these dimerization devices can of course also be used to link other functionalities such as toxins, marker enzymes, or enzymes with a prodrug-activating activity.

In obtaining exclusive hetero-dimer formation, the parallel-coiled coil strategy is most useful in combination with scFv fragments. A similar approach, albeit with Fab fragments, has been reported using the zippers from the transcription factor *fos* and *jun* (Kostelny et al. 1992). Since this strategy does not guarantee unique chain assembly of the Fab fragments, it seems less suitable than a strategy linking the scFv fragments, where the H and L chain of each binding site are already correctly linked. If $(Fab)_2$ fragments are desired, chemical crosslinking is probably the method of choice. With heterodimeric scFv fragments, it appears that certain helix-constructs are themselves prone to proteolysis in *E. coli* (Pack & Plückthun, in preparation). From the known crystal structure of the leucine zipper, however, fully synthetic helices can be designed that may be optimized with respect to these considerations (Pack and Plückthun, unpublished).

While a continuous chain linking two scFv fragments appears at first as an attractive idea, a 3D model with the binding sites at the same orientation as in a natural antibody immediately shows that such a linker will have to be of enormous length. An approach of this type has been reported for a CD4-scFv-C_K fusion (Traunecker et al. 1991), but the simple fusion of two scFv fragments which fold and are both simultaneously active has not yet been described. In crosslinking different molecules or cells, as opposed to binding to the same surface, shorter linkers may be sufficient, but the miniantibody designs discussed here take advantage of the fact that the C-terminus of the single chain Fv fragments need to come together, if the binding sites face away from each other. Thus, the miniantibody design is commensurate with the topology of the scFv fragments.

While recombinant Fv fragments or Fab fragments (Carter et al. 1992, Cumber et al. 1992) can be linked to form dimers using chemical crosslinking, preferably at cysteines introduced at the C-terminus, this requires a larger number of *in vitro* manipulations. This crosslinking technology can of course also be used with Fab′ fragments obtained by proteolysis or even whole antibodies. Yet, the miniantibody approach permits the assembly to take place in the *E. coli* cell, and one may then directly isolate dimers. It is not yet clear whether the dimerization handles are immunogenic. In future improvements of this design, the accessible (outside) portion of the helices might be "humanized" and taken from known human proteins. Furthermore, it will be necessary to study the pharmacokinetics of the miniantibodies to determine their spectrum of applications in therapy and

diagnostics. These molecules may perhaps constitute examples where hybrids from natural and scientifically designed proteins ultimately find their way into therapeutic principles.

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