The Pharmacology of Monoclonal Antibodies

Contributors

R. Balint, C.F. Barbas, R.D. Blumenthal, P. Carter, M. Chatterjee Chen, Y.-C. Jack, R.M. Conry, K.A. Foon, D.M. Goldenberg E. Haber, M. Hein, A. Hiatt, K. James, K.D. Janda, K. Karjalainen H. Kohler, J.W. Larrick, A.F. LoBuglio, N. Lonberg, G.E. Mark E.A. Padlan, S.H. Pincus, A. Plückthun, M.L. Rodrigues R.G. Rupp, M.N. Saleh, M.R. Shalaby, R.M. Sharkey A. Traunecker

Editors

Martin Rosenberg and Gordon P. Moore

Springer-Verlag
Berlin Heidelberg New York London Paris
Tokyo Hong Kong Barcelona Budapest

CHAPTER 11

Antibodies from *Escherichia coli*

A. PLÜCKTHUN

reprinted from:
A. Plückthun, Antibodies from Escherichia coli.
In: The pharmacology of monoclonal antibodies,
M. Rosenberg and G. P. Moore, Eds. (Springer
Verlag, Berlin, 1994), vol. 113, pp. 269-315.

A. Introduction

Antibody engineering is still at its very beginning. No matter what the goal of the study, it is likely that a number of different ideas and variants of the recombinant antibody will have to be tested. It is therefore useful to be able to make new variants of the antibody protein easily, reliably and fast. Mostly for historic reasons, the molecular biology of *Escherichia coli* is more advanced than that of any other species; in fact essentially all molecular cloning is done today with this bacterial strain. It is therefore attractive to carry out antibody expression with this bacterium as well.

This chapter will describe the molecular biology of antibody expression in *Escherichia coli*. Some of the problems encountered will be common to all protein expression in *E. coli*, and they will not be discussed in detail. As will become apparent, the unique problem of making antibodies in *E. coli* is not so much one of ribosomal biosynthesis of the polypeptide (and certainly not one of efficient transcription, as this problem is more or less solved), but one of efficient folding of the polypeptide in the cell or, for some applications, in vitro. For this reason, it will be necessary to discuss the peculiarities of the antibody protein structure: its domain-like organization, its conserved disulfide bonds and its distinct sequence variability, which all have an effect on expression.

Due to their glycosylation and their large size, the production of functional whole antibodies in *E. coli* is still impractical, and the relation of the particular antibody fragment chosen, its biophysical characteristics and the effects on expression (that is, folding efficiency) must also be analyzed. In this context, solutions of the bivalency problem uniquely suited for bacterial expression will be discussed.

The bacterial system offers the possibility to produce fragments of the antibody directly, which can be useful for applications in which only binding is required. Examples might be in vitro diagnostics, affinity chromatography, antigen stabilization or catalysis. In other instances, the desired final product may be a whole antibody, humanized or human, to be used, for example, in therapy. In such cases, it can still be advantageous to carry out all testing of variants of the antigen binding site with *E. coli* fragments and then transplant the finished version to the eukaryotic expression system of the whole

antibody. Finally, the easy availability of the recombinant product invites construction of many new molecules: hybrid proteins and antibodies with engineered metal binding sites, or with peptide tags for easy detection, or molecules with substantial alterations in the framework and quaternary structure.

Besides these "rational" alterations, *E. coli* permits a unique access to random approaches. Because of the unparalleled efficiency with which transformation with DNA or transfection by phages can be achieved in *E. coli*, many types of libraries of antibody variants can be assembled and screened by methods discussed below. With this background as a rationale, the emphasis of this article on the process of in vivo folding and assembly as the basis of all engineering and screening may become apparent.

B. Expression of Functional Antibody Fragments in *E. coli* by Secretion

I. General Overview

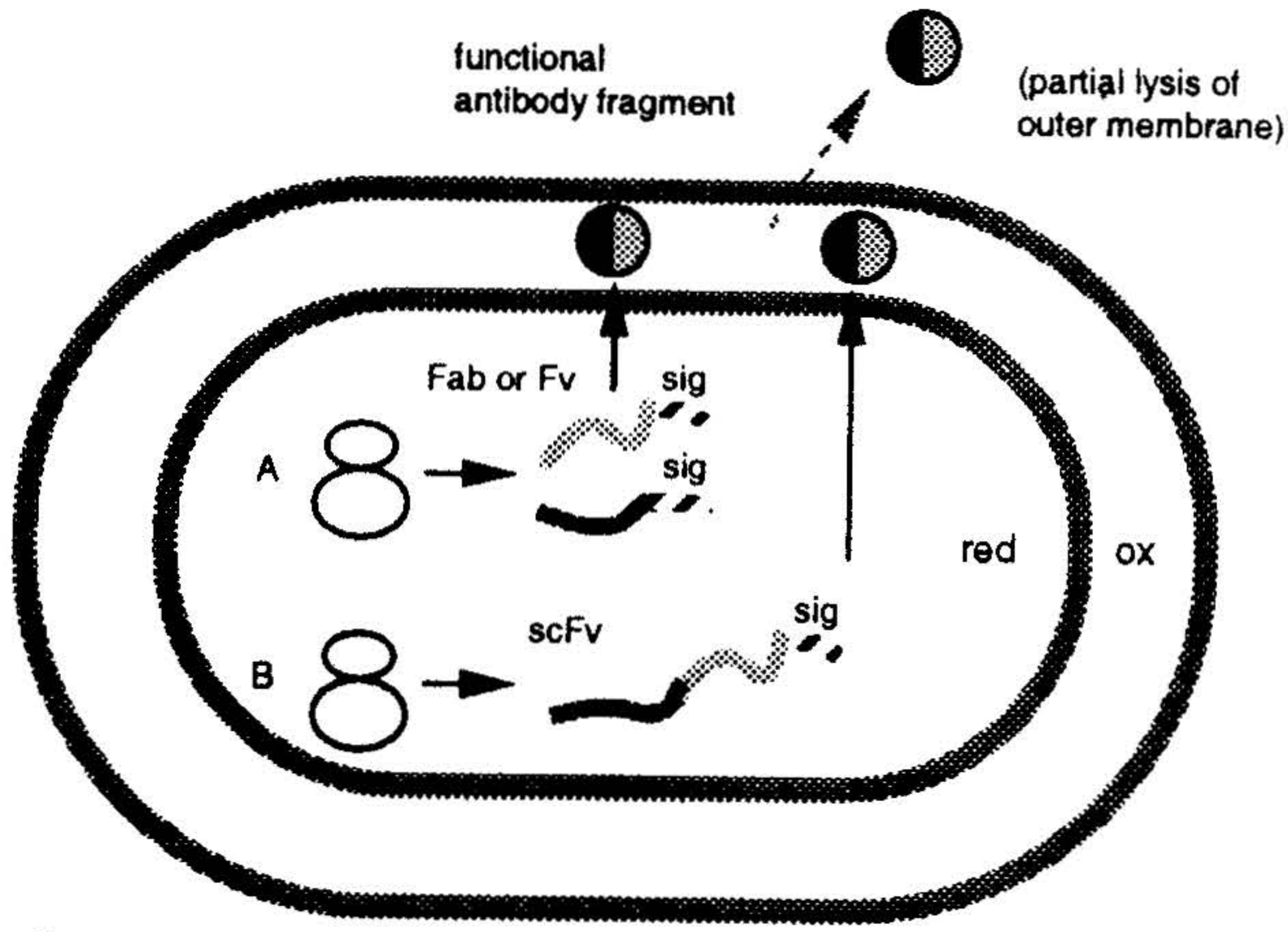
There are two basic strategies for obtaining recombinant antibodies and their fragments from *E. coli* (Fig. 1). The first is to produce inclusion bodies

Fig. 1a–c. The different expression strategies found to be useful for antibody production in *E. coli*. **a** Functional expression: In this case, the two chains making up the antibody combining site must be transported to the oxidizing milieu of the periplasm. In this compartment, there is a disulfide forming enzyme (DsbA) which allows the formation of the crucial intramolecular disulfide bonds, found in each domain and required for stability. *A* The pathway for two independent chains (as in a Fv or Fab fragment) is shown. Both are made as precursors containing a signal sequence and are then transported to the periplasm, where the signal sequence is cleaved off. There, folding, disulfide formation and assembly takes place. *B* Alternatively, both variable domains can be linked to form a continuous polypeptide chain (scFv fragment) which can then also be secreted.

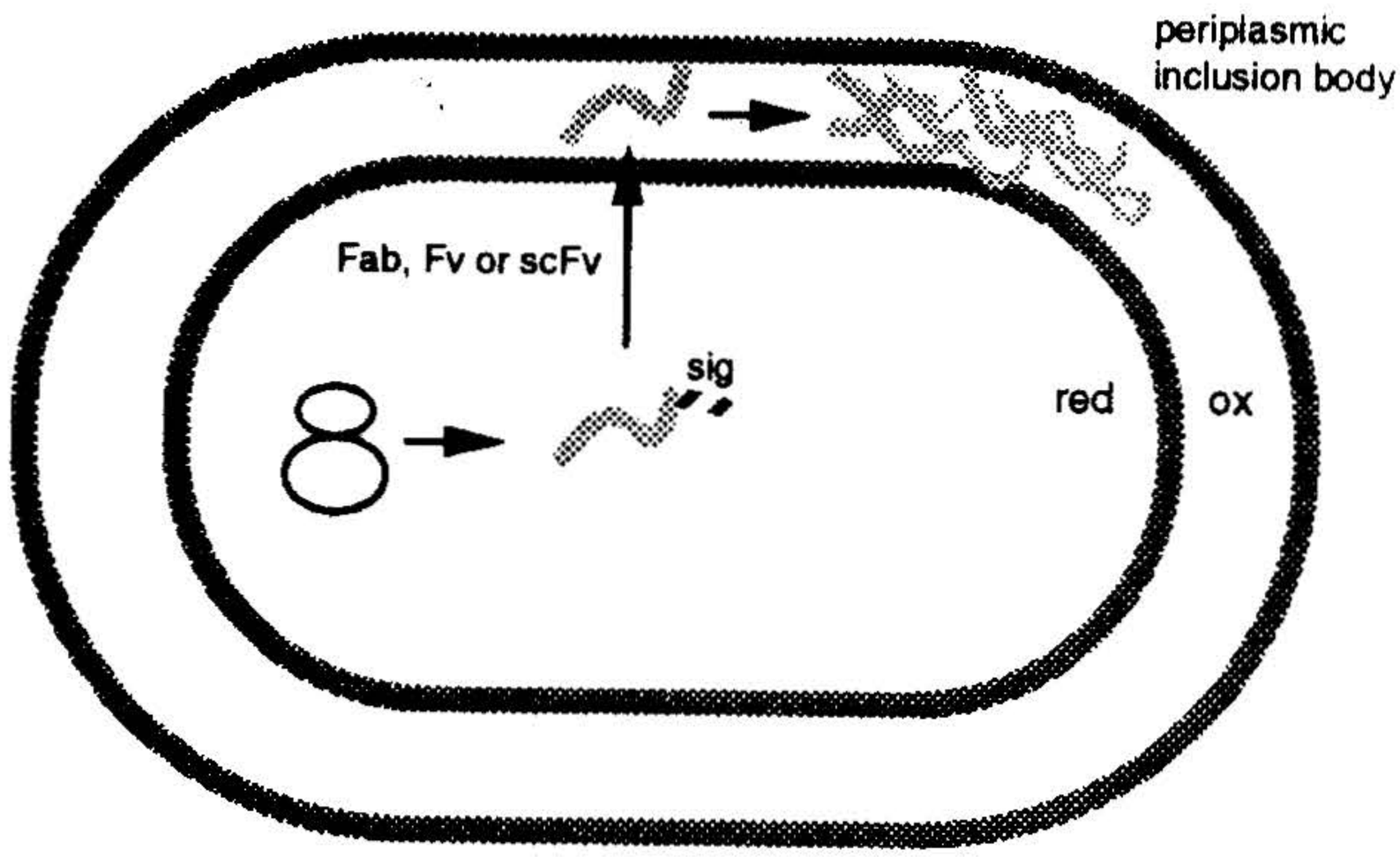
For reasons not understood in mechanistic detail, secretion of these proteins presents a stress to *E. coli* and results in leakiness of the outer membrane after some time. This appears to occur to some degree with all types of antibody fragments and signal sequences tested, but its magnitude depends on the primary sequence of the antibody. The phenomenon is observed more readily in shake flasks than in controlled fermentation conditions, where it can be minimized. To prevent leakage and periplasmic inclusion body formation, it is crucial to grow cells at low temperatures (e.g., 25°C).

b Periplasmic inclusion body formation. This is observed for many fragments, but has been exploited preparatively mostly for scFv fragments. This phenomenon is temperature-dependent and is most easily induced at a temperature of 37°C or higher. The protein is apparently transported, processed and then precipitates. The protein must be refolded in vitro as in **c**.

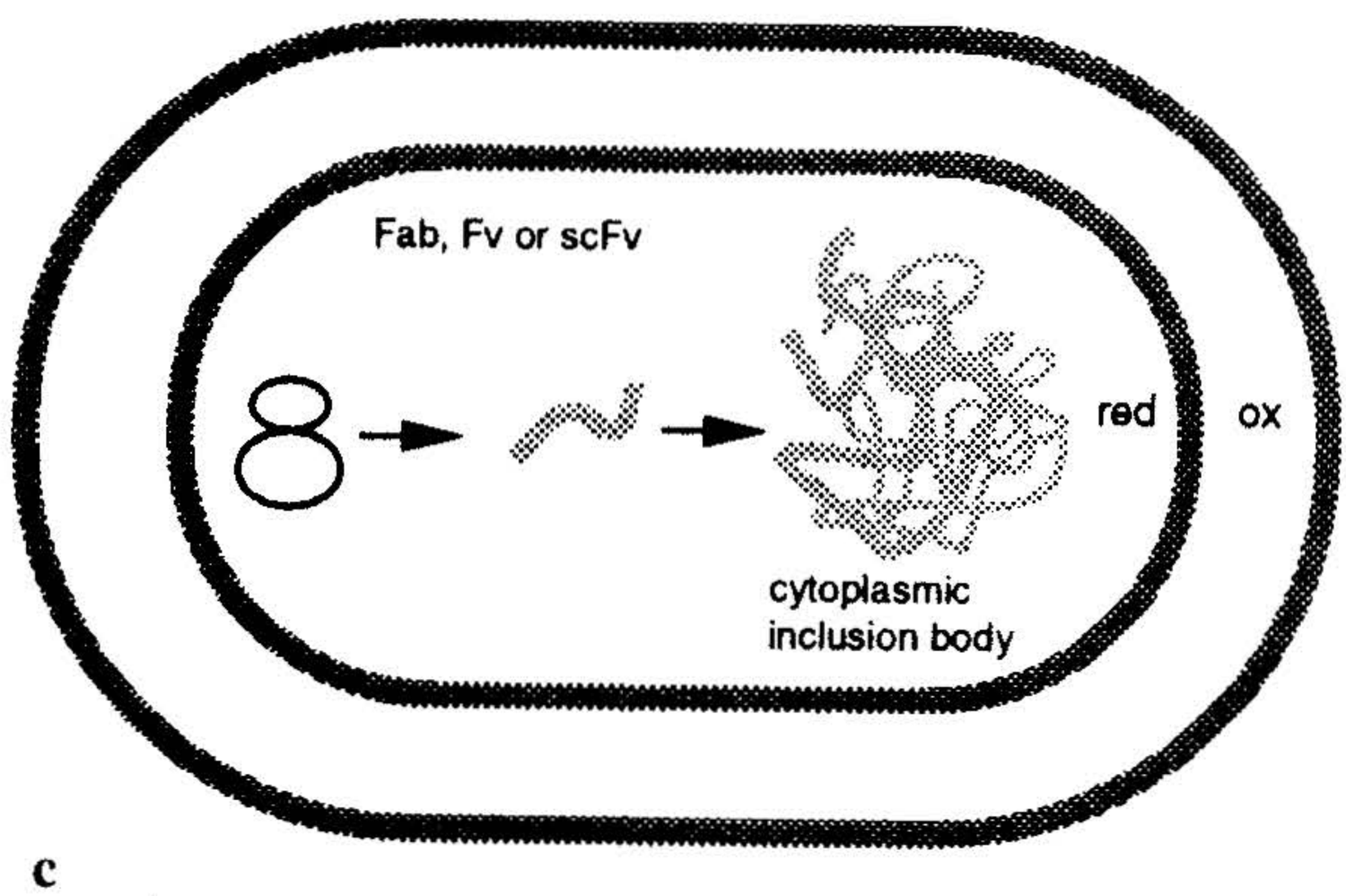
c Cytoplasmic inclusion body formation. In this case, the protein is expressed without a signal sequence under as strong a promoter and translation signal as possible. Inclusion body formation appears to be more successful at temperatures of 37°C and higher. The protein must be refolded in vitro


functional
antibody fragment
(partial lysis of
outer membrane)
Fab or Fv
sig
A
sig
red
ox
scFv
sig
B

a


periplasmic
inclusion body
Fab, Fv or scFv
sig
red
ox

b


Fab, Fv or scFv
red
ox
cytoplasmic
inclusion body

c

and refold the protein in vitro. The second is to imitate the situation in the eukaryotic cell and secrete the protein. With this method, completely functional antibody protein can be obtained (SKERRA and PLÜCKTHUN 1988; BETTER et al. 1988), and this strategy will be discussed first.

In *E. coli* a secretion machinery exists which leads to the transport of those proteins to the periplasmic space which carry a signal sequence (for a recent review, see PUGSLEY 1993). These are usually proteins which need to be outside of the "main" cell, for example, to degrade some biomolecules for easier uptake (e.g., peptidases, phosphatases). This secretion allows the protein to escape from the reducing environment of the cytoplasm, which in general, while perhaps not always (CABILLY 1989), appears to prevent normal disulfide formation (GILBERT 1990). Antibodies, as proteins which are normally secreted and equipped with disulfide bonds which are generally, but perhaps not for every primary sequence, necessary for stability (GLOCKSHUBER et al. 1992), ought to be secreted in bacteria, too, in order to reach a folded state identical to that formed in eukaryotes.

The *E. coli* secretion machinery directs the protein to the periplasm. There are specialized systems for transport out of the cell (PUGSLEY 1993), but they have not yet been used for high level production of foreign proteins (HOLLAND et al. 1990). It should be noted that, under conditions of high temperature and depending on the particular primary sequence and fragment of the antibody, a leakiness of the outer membrane is frequently observed with a variety of signal sequences (PLÜCKTHUN and SKERRA 1989). In these circumstances, periplasmic markers such as β-lactamase and alkaline phosphatase are also found in the medium, just as is the recombinant antibody protein. Nevertheless, some authors still refer to this phenomenon loosely as 'secretion to the medium" (see, e.g., TAKKINEN et al. 1991). This leakiness, whose molecular cause is still unknown, is possibly related to the formation of insoluble periplasmic protein from an aggregation process that occurs after the protein has been transported through the membrane (Fig. 1), but it may indicate the interference of the antibody with the transport of some crucial outer membrane component. After some time, the cells can also lyse completely, and this limits the production phase.

Periplasmic secretion has permitted the functional expression of a wide variety of antibody fragments with many antigen binding specificities. Because of the general importance and widespread application of this technology, it may be useful to discuss both the physiological limitations of the process in this chapter and the potential solutions to these problems. However, using this technology, it is already possible to conveniently obtain recombinant antibody fragments in sufficient amounts for essentially all studies.

II. Relation of Functional Secretion to Phage Libraries

An interesting and important consequence of the successful periplasmic folding of antibody fragments is the compatibility of the antibody folding

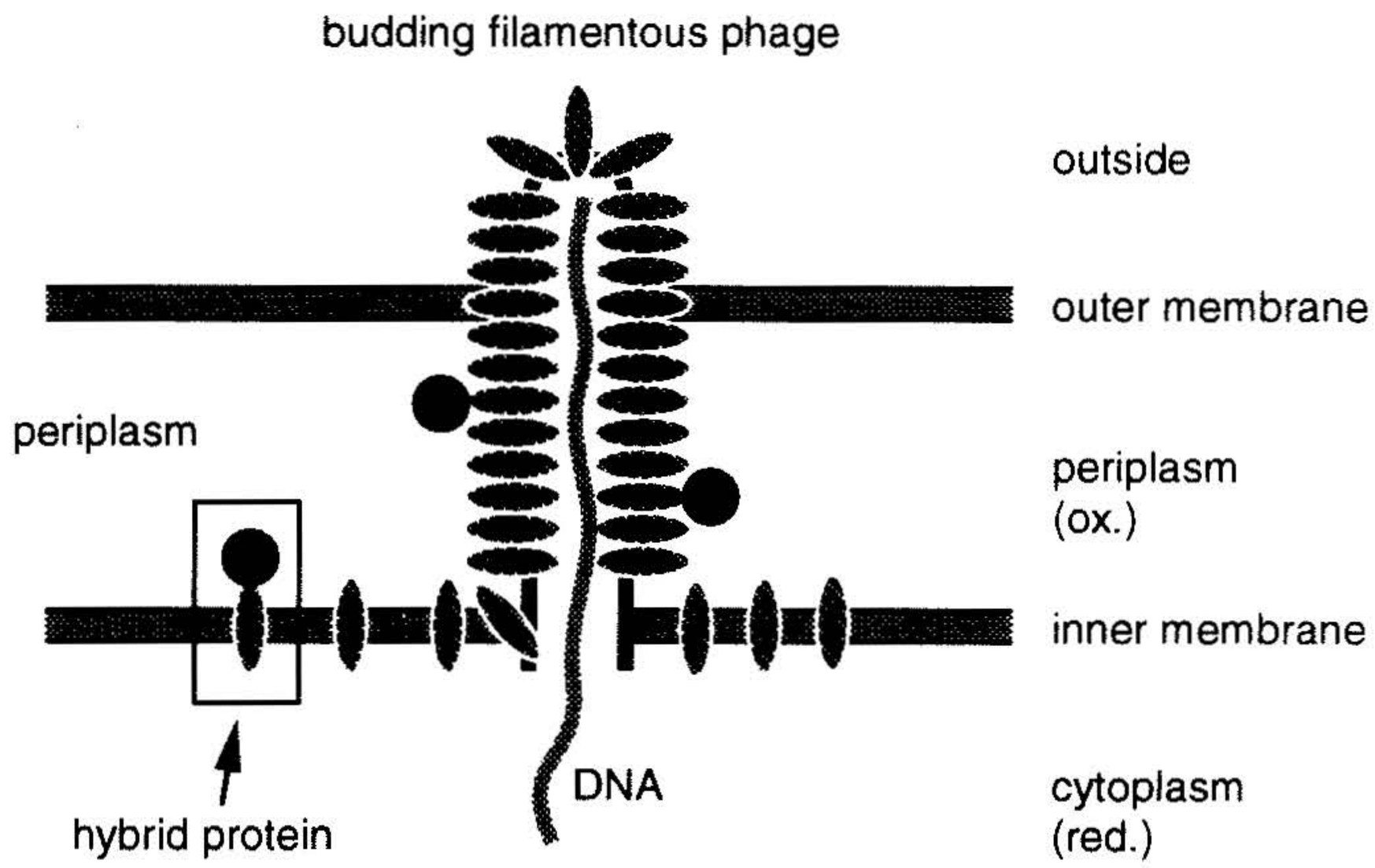


Fig. 2. The coating of a filamentous phage with hybrid proteins, present as intermediates in the inner membrane of *E. coli*

with display on the surface of a filamentous phage, such as M13 or fd. Since the NH_2-terminus of both the minor coat protein g3p (the product of gene III) and the major coat protein g8p (product of gene VIII) are probably exposed to the aqueous surrounding in the phage coat, NH_2-terminal extensions are still compatible with phage assembly (Fig. 2). This was first demonstrated for peptides displayed on the phage coat (SMITH 1985; PARMLEY and SMITH 1988; SCOTT and 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 NH_2-termini exposed to the periplasmic space. The budding phage then coats its DNA with these proteins waiting in the membrane. Consequently, any protein folding of fusion proteins consisting of NH_2-terminal antibody fragments and COOH-terminal phage coat proteins would take place in the periplasm, but while anchored to the inner membrane via the COOH-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. This has the important consequence of coupling genotype (the antibody gene on packaged phagemid) and phenotype (the displayed antibody fragment). Such experiments have been carried out with both Fab 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; KANG et al. 1991; CHANG et al. 1991; HOOGENBOOM et al. 1991; GARRARD et al. 1991; GRAM et al. 1992; BARBAS et al. 1992a) and will be discussed in more detail elsewhere in this volume. It is occasionally surmised that this process will automatically select for efficient folding and thus expression. However, while extremely poor expression will undoubtedly be selected against, it is unclear to what degree, at the extremely low expression levels of the minor phage coat proteins,

small differences in aggregation tendency of the displayed antibody fragment would be apparent.

The use of filamentous phage in antibody libraries must be contrasted with the use of phage λ (HUSE et al. 1989). In this case, the phage only delivers the DNA to the *E. coli* cell. In the λ phage genome, a plasmid constructed according to the principles laid out by SKERRA and PLÜCKTHUN (1988) and BETTER et al. (1988), is incorporated. Since production of viable phage would severely interfere with protein production (which of course requires healthy cells), the plasmid must be rescued after library formation before useful production is possible.

The display of proteins on the surface of *E. coli* has been described as well (FRANCISCO et al. 1992, 1993; KLAUSER et al. 1992; FUCHS et al. 1991), but as of yet no work with libraries has been reported. It remains to be seen how such screening methods would compare to the screening of filamentous phage libraries.

III. Description of the Secretion Process

At room temperature, a major portion of the Fv fragments and a significant fraction of the Fab fragments investigated in detail (SKERRA and PLÜCKTHUN 1991; KNAPPIK et al. 1993, unpublished) go to the native state (Fig. 3), but it is now clear that this portion crucially depends on the primary sequence of the variable domains. It was shown that the antibody fragments are processed correctly, contain their disulfide bonds, assemble to heterodimers and bind antigen with the same affinity as the normal antibody (SKERRA and PLÜCKTHUN 1988). The two chains of the Fv or Fab fragment therefore find

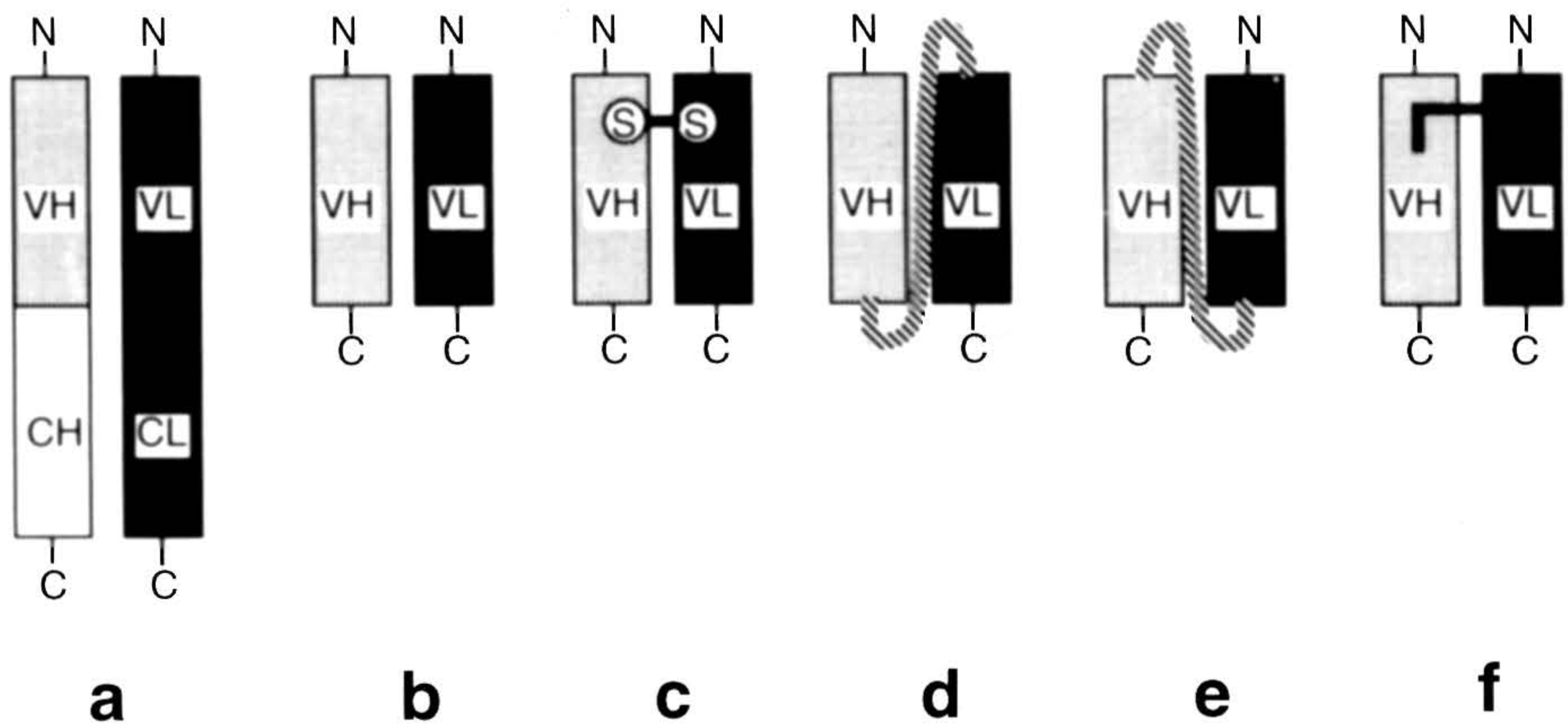


Fig. 3a–f. Monovalent fragments of antibodies functionally expressed in *E. coli*. **a** Fab fragment; **b** Fv fragment; **c** disulfide-linked Fv fragment; **d** single chain Fv fragment with the orientation V_H-linker-V_L; **e** single chain Fv fragment with the orientation V_L-linker-V_H; **f** Fv fragment which has been stabilized by chemical cross-linking after purification. (See GLOCKSHUBER et al. 1990a)

each other even if they are not covalently linked, as they do in eukaryotes. This has now been demonstrated for a variety of Fv fragments (see, e.g., SKERRA and PLÜCKTHUN 1988; WARD et al. 1989; GLOCKSHUBER et al. 1991; MCMANUS and RIECHMANN 1991; ANTHONY et al. 1992; STEMMER et al. 1993a) and Fab fragments (see, e.g., BETTER et al. 1988; PLÜCKTHUN and SKERRA 1989; ANAND et al. 1991a; CARTER et al. 1992; BARBAS et al. 1992b; BETTER et al. 1993), demonstrating the generality of the method. In conclusion, the antibody binding site of the fragments produced in bacteria is functionally identical to the natural antibody (see also Sect. 1). Low bacterial growth temperature is the most efficient method to minimize periplasmic aggregation and maximize the yield of folded antibody protein (SKERRA and PLÜCKTHUN 1991; KNAPPIK et al. 1993). This may have to do with the stability of folding intermediates and the rate of protein biosynthesis or secretion, both of which are functions of temperature, but the relative importance of these phenomena is not yet clear.

From an investigation of the limiting step in the process of secreting antibody fragments, it was concluded (SKERRA and PLÜCKTHUN 1991) that, if a vector with strong transcription and translation initiation signals is used, it is periplasmic folding and/or assembly which limits the level of functional expression. During antibody folding, the insoluble periplasmic protein is formed as a by-product, presumably via the aggregation of an intermediate. This conclusion was arrived at as follows: With increasing promoter strength, the amount of correctly folded antibody does not increase significantly. The insoluble, correctly processed antibody protein increases, and only at the highest promoter strength is there a significant sign of precursor. This insoluble protein was shown, at least in one case (GLOCKSHUBER et al. 1992), to be accessible to externally added proteases after producing spheroblasts under conditions in which soluble cytoplasmic precursor was not degraded. One may conclude that the signal sequence is cleaved off and that part of the protein folds correctly to produce fully functional antibody fragments, although some part of it does not achieve the native state and instead aggregates and precipitates (Fig. 1).

In the antibody McPC603 (PERLMUTTER et al. 1984; PLÜCKTHUN 1993a), the Fv and Fab fragments are produced at about similar amounts on the ribosome from the same vector, and the total amount of protein produced is comparable (SKERRA and PLÜCKTHUN 1991; KNAPPIK et al. 1993). However, the amount of functional protein is greater for the Fv fragment. This finding has suggested that particular folding problems in the C_H1 or C_L domains may be responsible for the less efficient folding of the Fab fragment. Alternatively, the presence of the constant domains may just potentiate folding problems in the variable domains. There is evidence supporting the latter view, as different variable domains in the same Fab vector fold in different proportions (KNAPPIK and PLÜCKTHUN, unpublished). This phenomenon may be masked if an Fv fragment is particularly prone to proteolysis (SCHWEDER and PLÜCKTHUN, unpublished).

While protein folding is an exergonic and spontaneous reaction, it is now clear that proteins exist which guide this process to prevent the side reaction of aggregation (JAENICKE 1993). No general periplasmic molecular chaperone has been unambiguously identified at this time (see, e.g., PUGSLEY 1993). There is no evidence, nor is it likely, that cytoplasmic molecular chaperones such as GroEL or DnaK (JAENICKE 1993) have any direct influence on periplasmic protein folding processes. They may, however, help the assembly of phage particles displaying the antibody under certain conditions (SÖDERLIND et al. 1993), and phages are known to require chaperone assistance in their assembly (ZEILSTRA-RYALLS et al. 1991), but there is no evidence to suggest that the chaperone influences antibody folding. Cytoplasmic chaperones may have an indirect effect on cell stability.

IV. The Role of Periplasmic Protein Folding

There is great variation in the literature about the reported efficiency of the secretory expression method for antibodies. This fact has to do with variations among vectors, procedures and quantification and the use of different fragments of antibodies varying in sequence. While the problem is far from being understood and still further from being solved, enough experiments have now been carried out to at least attempt some kind of correlation.

From a variety of experiments (CARTER et al. 1992; KNAPPIK and PLÜCKTHUN, unpublished), evidence is accumulating that the primary se-

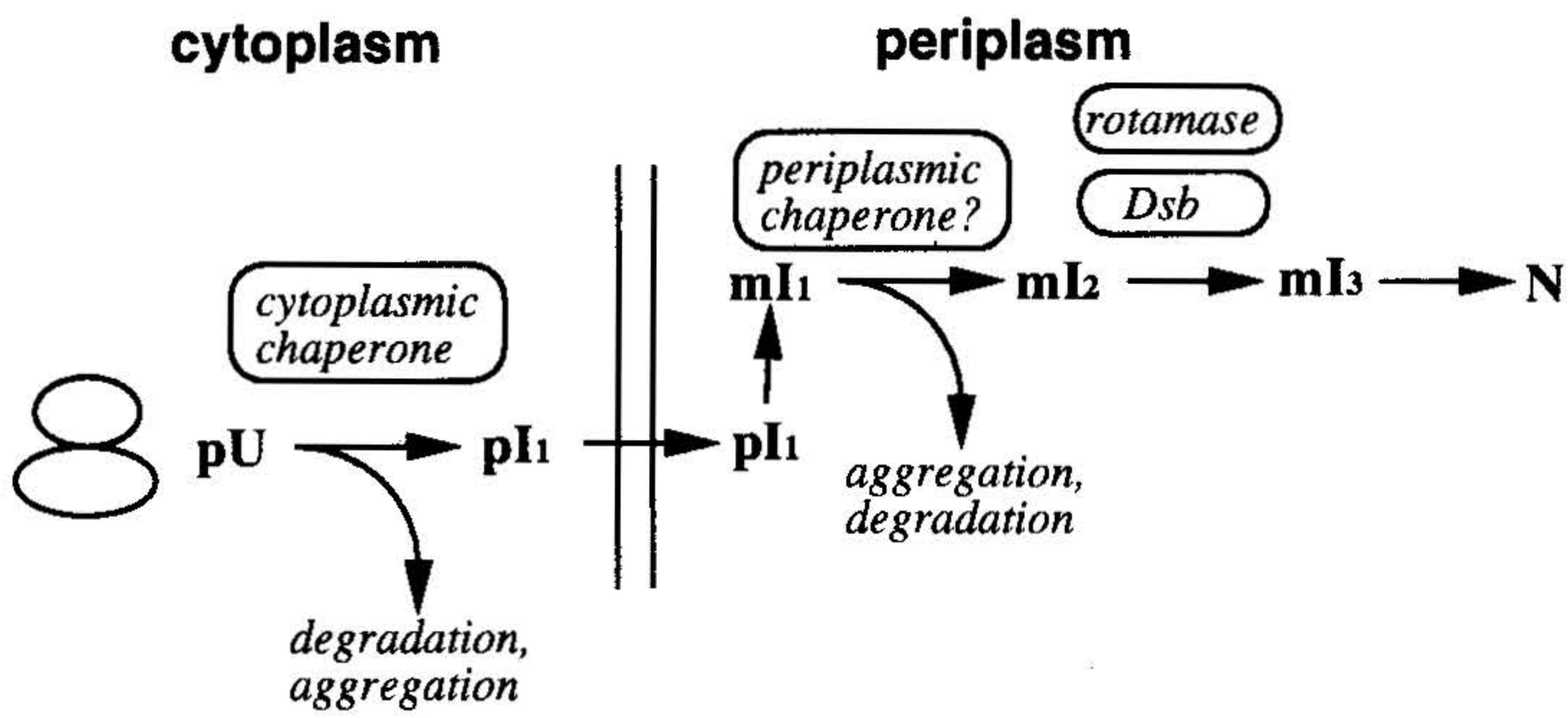


Fig. 4. Hypothetical folding pathway of a secreted protein. The protein is made on the ribosome as an unfolded precursor (*pU*) and probably kept in a transport-competent state (pI_1) by association with an as yet unidentified cytoplasmic factor (*chaperone*). This factor presumably prevents premature folding. It is unknown whether a fraction of the protein is degraded on its way to the membrane. After transport, the signal sequence is cleaved to give the mature folding intermediate mI_1, which must then fold via other hypothetical intermediates (mI_2, mI_3) to the native state *N*. During periplasmic folding, as yet unidentified periplasmic chaperones may act on the protein, and the disulfide forming activity *Dsb*A acts on the antibody. It is unknown whether the resident proline *cis-trans* isomerase (*rotamase*) acts on the antibody. Overexpression of rotamase and DsbA do not seem to change the amount going to N, and thus the diversion to aggregates appears to happen before these steps, or at least to be independent of their extent

quence of the antibody plays a decisive role. The primary sequence of the antibody determines the critical partitioning of the protein intermediates (presumably after transport) between folding to the native structure, aggregation and degradation (Fig. 4). This deduction requires constructs to be compared with each other which differ in nothing else but the primary sequence, i.e., the same type of fragments (Fab, Fv, single chain Fv) in exactly the same vector. As a case in point, an Fab fragment of a humanized antibody gave much more favorable (10- to 50-fold) partitioning between folding and aggregation than the chimerized version of the same molecule containing the mouse variable domains (CARTER et al. 1992).

Other antibody fragments may conceivably be prone to proteolysis and thus not reach the expression limit possible by folding. In poorly designed vectors, it may of course also be possible that other steps, such as transcription or translation, are limiting and expression is so low that insoluble protein is never seen. In low expression systems, it is thus possible that an improvement of these processes will increase the overall yield of folded protein (STEMMER et al. 1993a). It should be generally feasible, however, to reach the limit set by protein folding with suitable vectors (see below and, e.g., SKERRA and PLÜCKTHUN 1991), and it was shown that with such vector systems the antibody protein can be one of the most prominent soluble proteins in the cell, if it has a sequence and structure which allows efficient folding (KNAPPIK and PLÜCKTHUN, unpublished; CARTER et al. 1992).

V. Catalysis of Periplasmic Protein Folding

1. Disulfide Bond Formation

The observed difference in folding between the Fv and the Fab fragment of the same antibody (SKERRA and PLÜCKTHUN 1991) led to the question of whether particular structural features in the constant domains can be delineated which might be responsible for this difference, perhaps by leading to particularly slow folding steps of the Fab fragment. Two types of slow processes in protein folding have been discovered which can be pinpointed to particular chemical events (FISCHER and SCHMID 1990): proline *cis-trans* isomerization and disulfide formation and isomerization. It is now clear, however, that these are not the only slow events and that noncovalent rearrangements of the protein during folding, once the protein has already attained a native-like structure, can be slower still and thus can be rate determining. Unfortunately, the rate determining step for most antibody fragments is not known and, again, may well depend on the primary sequence.

The importance of proline *cis-trans* isomerization and disulfide isomerization can be tested by mutagenesis experiments and by catalysis with specific proteins in vitro and in vivo. Such experiments have been carried out with different fragments of the antibody McPC603 (SKERRA and PLÜCK-

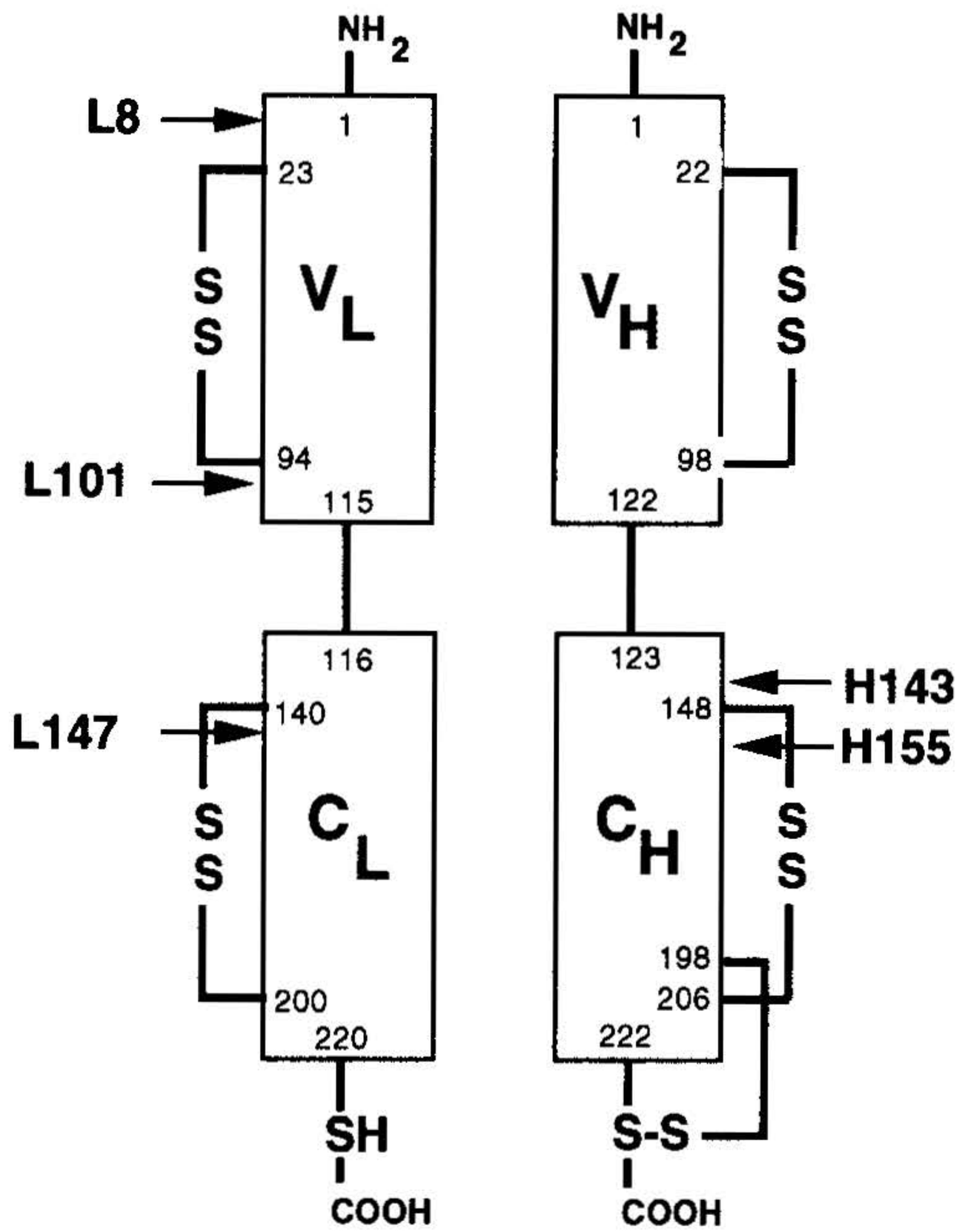


Fig. 5. Cysteine residues and *cis*-peptide bonds adjacent to proline residues in the Fab fragment of the antibody McPC603, a mouse IgA

THUN 1991; KNAPPIK et al. 1993) in *E. coli* and a single chain Fv-toxin fusion protein in vitro (BUCHNER et al. 1992a).

The Fab fragment of McPC603, a mouse IgA, carries five disulfide bonds (SATOW et al. 1986). One consensus S-S bond is present in each domain linking the two β-sheets, and there is an extra one in C_H1, which is a feature of mouse IgA (SATOW et al. 1986; COCKLE and YOUNG 1985) (Fig. 5). Removing this additional disulfide creates an Fab fragment which is fully functional but is not obtained at significantly higher yield (SKERRA and PLÜCKTHUN 1991), although the removal of two cysteines might at first be expected to diminish the possibilities for incorrect disulfide linkages. The light chain of this Fab ends in a free cysteine probably linked to the other Fab in the mouse IgA (ABEL and GREY 1968), but similarly, its removal does not change the partitioning of Fab between folded and aggregated protein. If the C_H1 domain of an IgG1 is introduced instead of the C_H1 of the mouse IgA, a covalent link is obtained between H and L, yet again at about the same level of correctly assembled protein. These data are consistent with the idea that disulfide formation does not limit the periplasmic folding process.

The formation of disulfide bonds is known to occur in the bacterial periplasm (POLLITT and ZALKIN 1983) (Fig. 4), and recently an enzymatic system has been discovered which is responsible for it (BARDWELL et al.

1991, 1993; KAMITANI et al. 1992). Briefly, the protein DsbA, itself containing a reversibly opening disulfide bond, is involved in the formation and perhaps rearrangement of disulfide bonds of the proteins to be folded after they have reached the periplasmic space. There has been some debate about whether disulfide rearrangement involving stable intermediates (as opposed to the serial formation of only correct disulfide bonds) is a physiologically relevant reaction (CREIGHTON 1978; WEISSMAN and KIM 1991; GOLDENBERG 1992). In the best investigated case in vitro, that of the bovine pancreatic trypsin inhibitor BPTI, disulfide formation occurs in a very late state of folding, after the tertiary fold is essentially complete (WEISSMAN and KIM 1991).

In the case of the antibody domains, the mechanistic details of disulfide formation have remained unclear. In their natural environment (the B cell or plasma cell) antibodies develop disulfide links in the endoplasmic reticulum (BERGMANN and KUEHL 1979), catalyzed by the eukaryotic disulfide isomerase (FREEDMAN et al. 1989). The conserved intradomain disulfide, however, is completely buried, and it seems that its formation must occur at an early step in folding.

Using a *dsbA*$^-$ deletion mutant, no formation of Fv fragment could be observed in the bacterial periplasm (KNAPPIK et al. 1993). Fv formation could, however, be restored by plasmid-encoded DsbA. These experiments show that bacterial DsbA takes part in the formation of active Fv fragment, and is *required* for this assembly.

An obvious question was therefore whether the overexpression of DsbA would increase the amount of active Fv, single chain Fv (scFv) or Fab fragment. For the antibody McPC603, for which such an experiment was carried out, the answer was negative (KNAPPIK et al. 1993). This is consistent with the results from the experiments on removing disulfides by mutagenesis (SKERRA and PLÜCKTHUN 1991), and it suggests that aggregation of the periplasmic protein occurs either before disulfide formation or is at least independent of its extent. This experiment does not, however, automatically lead to the generalization that disulfide formation may never be limiting for any fragments or fusion protein. There might conceivably be cases in which overexpression of a disulfide formation catalyst and/or the inclusion of redox couples (i.e., reduced and oxidized glutathione at various proportions) might make a difference. However, in the investigated cases, the aggregation phenomena seemed to be independent of the extent of disulfide formation and perhaps to precede it (Fig. 4).

In vitro, a strong dependence of folding rates and yields on disulfide formation has been seen for antibody folding (HABER 1964; ROWE and TANFORD 1973; ROWE 1976; GOTO and HAMAGUCHI 1979, 1982, 1986; GOTO et al. 1988; HUSTON et al. 1991; BUCHNER et al. 1992a,b; BUCHNER and RUDOLPH 1991). Nevertheless, different scFv fragments seemed to show rather different requirements for the type of oxidative folding: the method most frequently used for disulfide containing proteins, simultaneous oxida-

tion and refolding (by dilution from denaturant into a buffer containing a redox couple) with or without prior formation of mixed disulfides, was not always successful (summarized in HUSTON et al. 1991). Some antibody Fv and scFv fragments require oxidative formation of disulfide bonds already in the completely denatured state, suggesting problems with accessibility of the cysteines (HOCHMAN et al. 1976; GLOCKSHUBER et al. 1992).

In conclusion, the conserved disulfides of the variable domains, which are important for structural integrity (GLOCKSHUBER et al. 1992), can be formed in vitro and in vivo (in eukaryotes and bacteria) and they do not seem to constitute the reason for the amount of aggregation which may accompany antibody expression in bacteria.

2. Proline *cis-trans* Isomerization

Another reaction which can be the slow step in protein folding is proline *cis-trans* isomerization (BRANDTS et al. 1975). The peptide bond contains partial double-bond character and thus presents a significant barrier to rotation. Two stable configurations exist, but for all amino acids except proline, the *trans* configuration (labeled with respect to the two C_α atoms) avoids steric crowding, whereas for proline, the difference is marginal (summarized in STEWART et al. 1990). Proline *cis-trans* isomerization can be a slow step of protein folding in vivo, even of antibody domains (GOTO and HAMAGUCHI 1982; LANG and SCHMID 1988; BUCHNER et al. 1992a). However, even for very slow folding proteins containing *cis* prolines, there may be packing rearrangements that occur still more slowly than proline *cis-trans* isomerization and thus constitute the rate-determining steps.

There are two unrelated, ubiquitous classes of proteins with proline-*cis-trans* isomerase activity (FISCHER and SCHMID 1990; TRANDINH et al. 1992). So far, actual demonstration of their involvement in folding in vivo is still lacking, but their acceleration of folding in vitro has been demonstrated for numerous substrate proteins. *E. coli* has two such enzymes, one in the cytoplasm, and one in the periplasm (LIU and WALSH 1990; HAYANO et al. 1991).

Antibodies contain *cis* prolines both in their constant and in their variable domains (Fig. 5). In the particular case of the Fab fragment of the mouse IgA McPC603, there are five of them (SATOW et al. 1986) (two in V_L at L8 and L101, one in C_L at L147 and two in C_H1 at H143 and H155). Thus, the Fv fragment would contain two *cis* bonds and the Fab fragment all five. The observation that the Fab fragment and the Fv fragment of the same antibody fold to various efficiencies in the periplasm, whereas they are produced in about the same amount on the ribosome, made it worthwhile to test proline *cis-trans* isomerization as a possible cause for the different behavior of Fv and Fab fragments.

For this purpose, a loop in the C_H1 domain at the opposite end of the molecule from the binding site was altered from its wild-type sequence

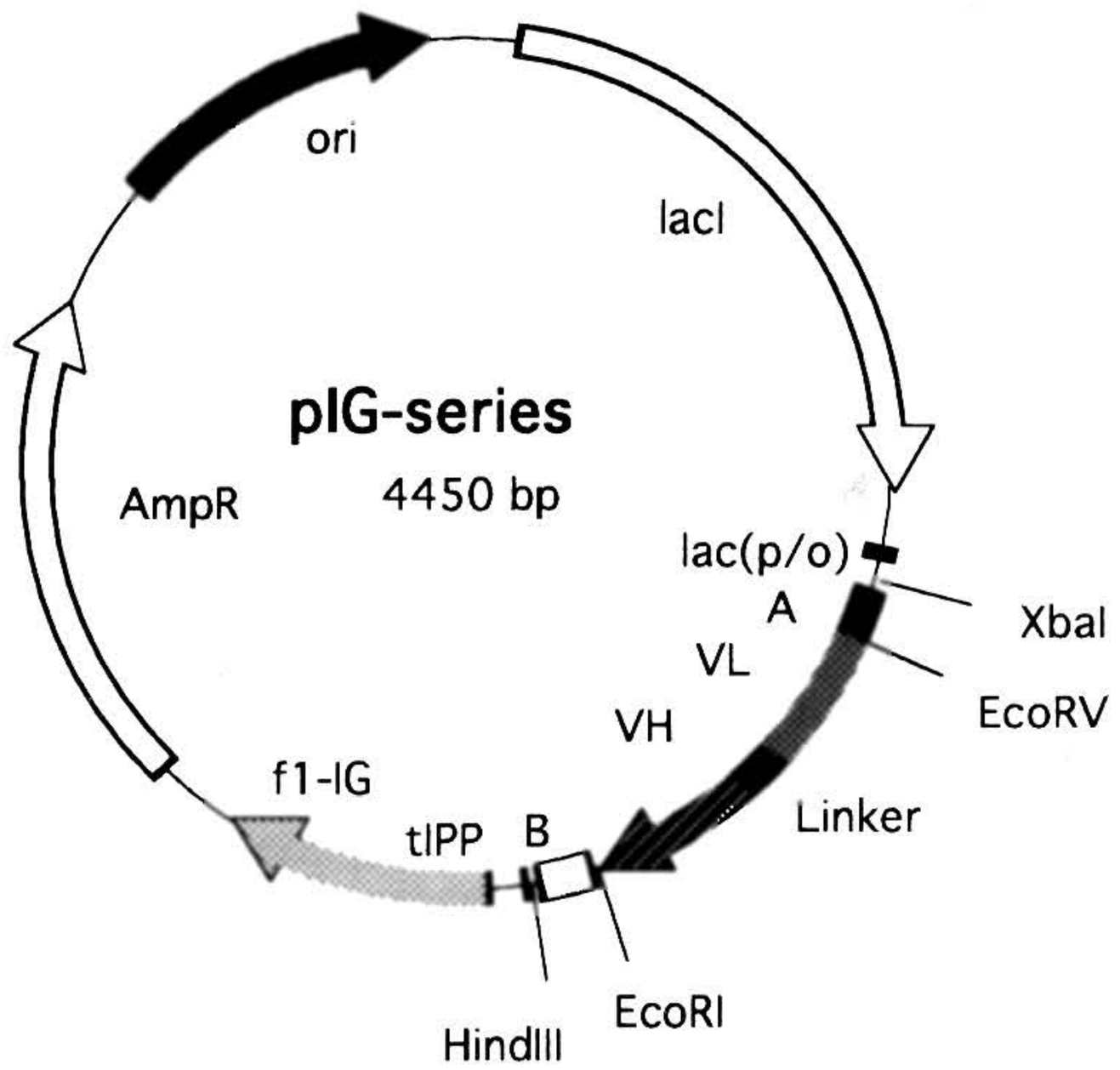
(containing two *trans*-prolines and one *cis*-proline) to a loop containing only 1 *trans*-proline, but without dramatic effect on periplasmic folding (SKERRA and PLÜCKTHUN 1991). Since the putative cause of aggregation might be in any of the *cis*-prolines, the *E. coli* periplasmic proline *cis-trans* isomerase was also overexpressed together with the Fv fragment, or both versions of the scFv fragment (V_H-linker-V_L and V_L-linker-V_H) or the Fab fragment (KNAPPIK et al. 1993). There was no effect on the yield of folded protein, with the possible exception of one of the scFv fragments. In this particular case, V_L was preceded by the linker and perhaps isomerization of the peptide bond at L8 was sterically more hindered than in any other fragment. Nevertheless, there is no evidence that proline *cis-trans* isomerization has a limiting role on the folding of these antibody fragments in *E. coli*. This conclusion is also not changed by overexpressing E. coli disulfide isomerase DsbA together with proline *cis-trans* isomerase (KNAPPIK et al. 1993).

It appears therefore that neither proline *cis-trans* isomerization nor disulfide isomerization can be held responsible for the absence of quantitative folding of antibody fragments in the bacterial periplasm. Rather, aggregation events which occur before these reactions or are at least independent of their extent appear to be the cause (Fig. 4). Nevertheless, periplasmic functional expression is possible and provides a fast, convenient and versatile method to directly obtain folded antibody in quantities sufficient for essentially any experiments, up to grams per liter in fermentation (CARTER et al. 1992).

VI. Design of Secretion Vectors

The previous discussion has emphasized that it is the periplasmic folding process which appears to limit the expression level of functional antibody protein. Therefore, it is not necessarily useful to choose as high an expression level as possible. Instead, it is crucial that expression is under the control of a repressible promoter, since secretion (or the concomitant production of some insoluble periplasmic protein) appears to stressful for the cells, which may respond with poor growth, plasmid loss, and, in extreme cases (observed for a single chain T cell receptor; WÜLFING and PLÜCKTHUN, unpublished), complete rearrangement of plasmids. Many different antibody secretion vectors have been designed by now, but they all essentially follow the principles laid out by SKERRA and PLÜCKTHUN (1988) and BETTER et al. (1988). Phages use the *E. coli* machinery to make proteins in just the same way. Therefore, they must contain similar expression cassettes. This is true for phage λ, in which the phage is merely used for transfecting the genetic information efficiently, and for filamentous phages (see above), in which a fusion protein is made from the antibody and a coat protein, using periplasmic secretion as an intermediate.

In a walk around the vector (Fig. 6), the considerations leading to the choice of promoter of the recombinant immunoglobulin will be discussed


ori
lacI
pIG-series
4450 bp
AmpR
lac(p/o)
A
XbaI
VL
EcoRV
VH
f1-IG
Linker
tIPP
B
EcoRI
HindIII

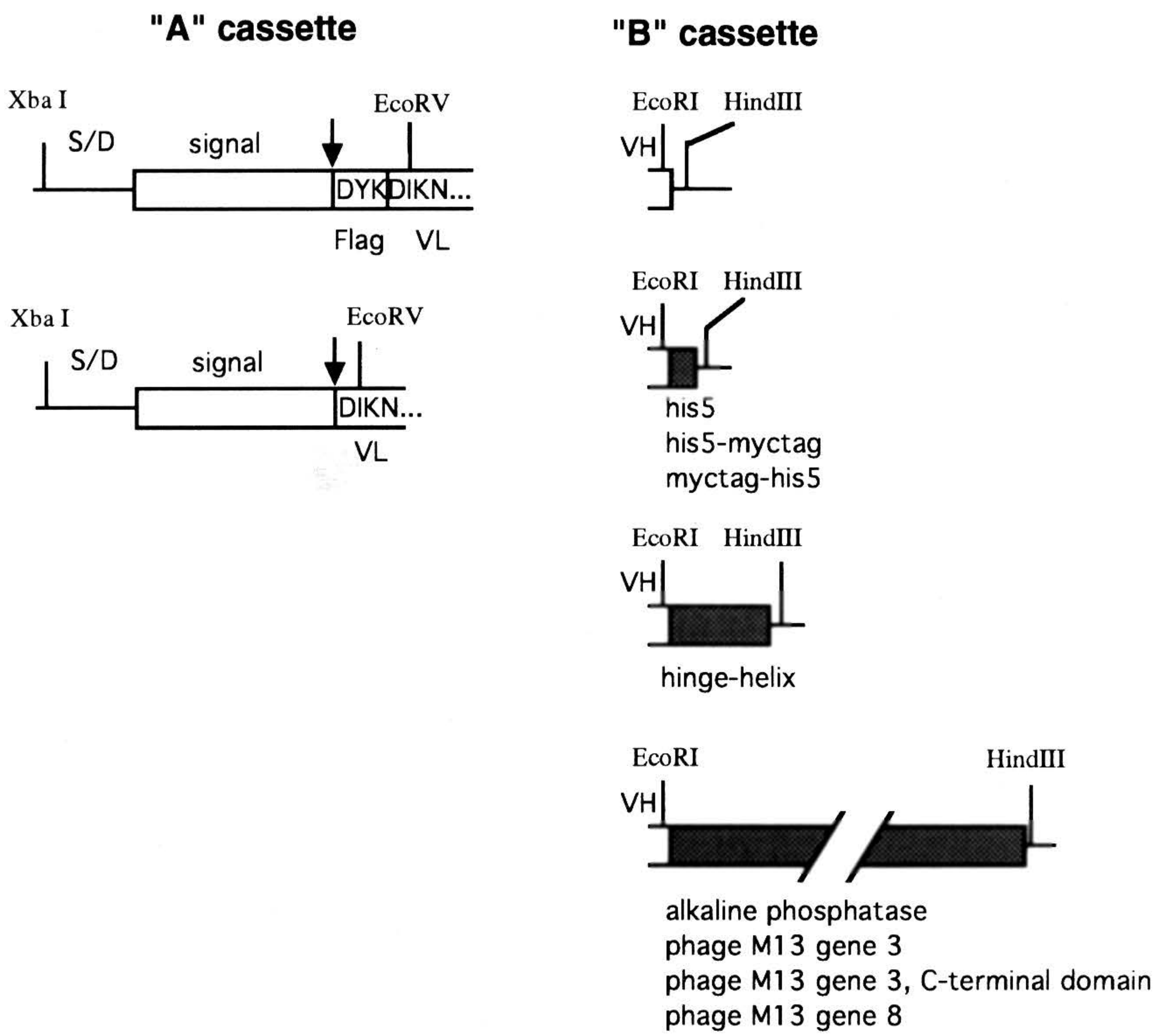

"A" cassette
"B" cassette
Xba I
EcoRV
S/D
signal
DYKDIKN...
Flag VL
Xba I
EcoRV
S/D
signal
DIKN...
VL
EcoRI HindIII
VH
EcoRI HindIII
VH
his5
his5-myctag
myctag-his5
EcoRI HindIII
VH
hinge-helix
EcoRI
HindIII
VH
alkaline phosphatase
phage M13 gene 3
phage M13 gene 3, C-terminal domain
phage M13 gene 8

Fig. 6A

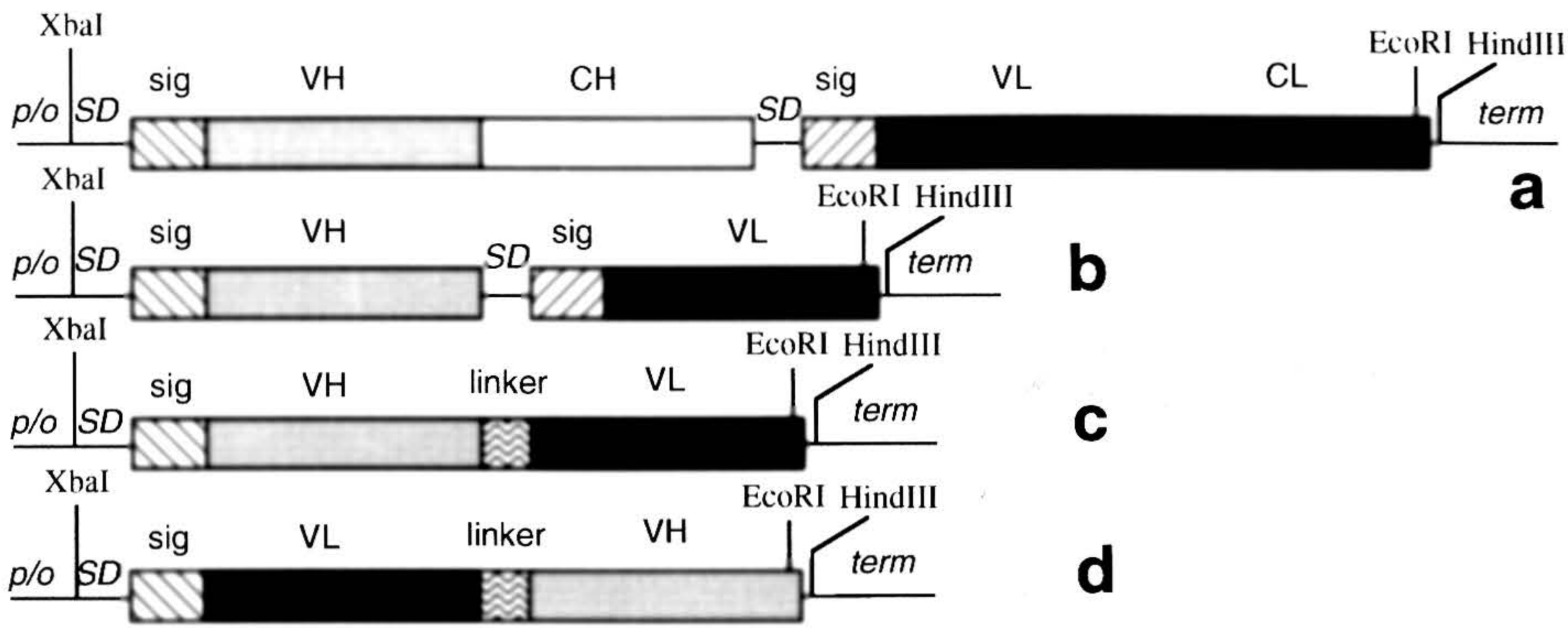


Fig. 6A,B. Secretion vectors for antibody expression in *E. coli*. As an example, a series of improved vectors suitable for expressing single chain Fv (scFv) Fv and Fab fragments is shown (L. GE, A. KNAPPIK and A. PLÜCKTHUN, unpublished). These vectors incorporate restriction sites within the antibody gene for convenient PCR cloning and cassettes for detection, purification and phage display.

A The design is very modular, allowing easy exchange of antibiotic resistance, the fragment of the antibody and the *A* and *B* cassettes.

The *A cassette* contains a bacterial signal sequence, directly fused to the mature antibody part or preceded by only three additional *Flag* amino acids, which can be detected with extremely high specificity and sensitivity using a Ca^{2+}-dependent antibody (KNAPPIK et al., unpublished) using a much shorter epitope than originally presumed to be necessary (PRICKETT et al. 1989).

The *B cassette* may be used to introduce a COOH-terminal purification or detection tag, to fuse a hinge and helix for dimerization, to fuse a phage gene for surface display or an enzyme for easy detection of the antibody.

PCR of antibody genes can be carried out with primers carrying extensions using *Eco*RI and *Eco*RV sites, which are both rare in antibody sequences.

B The schematic arrangement of genes for functional expression. Since in this case both chains of the antibody must be secreted to the same periplasmic space to assemble, they must either be produced as two different secreted protein chains in the same cell (*a,b*) or they must be linked via a peptide linker (*c,d*). It is advantageous to express the independent chains of the Fab fragment (*a*) or the Fv fragment (*b*) in a dicistronic operon (*a,b*), as discussed in the text. Two different orientation of the scFv fragment are shown which have both been shown to function (*c,d*). *p/o* denotes a promoter/operator structure; *SD*, a Shine-Dalgarno sequence; and *term*, a transcription terminator

first. While it should be strong, it needs to be, first and foremost, very tightly regulated. Two natural promoters have proven to be particularly useful in this respect, the first being the *lac* promoter, which is regulated not only by the *lac* inducer IPTG, but also by glucose (BECKWITH and ZIPSER 1970). The second is the *pho*A promoter (WANNER 1987), which is turned off in the presence of phosphate. The latter is more useful in reproducible fermentations, but perhaps less so on a laboratory scale, since the initial phosphate must be precisely calculated to run out at a particular point. A third system, which is completely tight and has been shown to be useful for T cell receptor expression, is that of invertible promotors (WÜLFING and

PLÜCKTHUN, 1993; PODHAJSKA et al. 1985), using phage λ integrase for inverting a cassette in which any promotor can be placed.

Less suitable are promoters such as *pL* when coupled with the thermolabile cI857 repressor, since they have to be induced at high temperatures. This is counterproductive with regard to correct folding (COLCHER et al. 1990; GIBBS et al. 1991), which occurs more efficiently at low temperature (TAKAGI et al. 1988; GLOCKSHUBER et al. 1990a; SKERRA and PLÜCKTHUN 1991), and it requires that the temperature is shifted back to room temperature in the expression phase.

The translation initiation region is usually taken from a well expressed protein and it might be taken from the same bacterial gene as the signal sequence. The principle of a mini-cistron preceding the actual antibody gene or genes, themselves arranged in two cistrons in the case of an Fab or Fv fragment, has been found useful (SKERRA et al. 1991; SCHONER et al. 1990). Despite intensive research (summarized in MCCARTHY and GUALERZI 1990), there is still only incomplete rational understanding of efficient translation initiation, especially the positive or negative effect played by secondary structure of the mRNA, which on the one hand prevents translation and on the other hand protects the mRNA from degradation (see, e.g., EHRETSMANN et al. 1992). Therefore, pragmatic approaches using well expressed *E. coli* genes as a framework still prevail. At low expression levels transcription and translation may be limiting (STEMMER et al. 1993a).

The signal sequence directs the antibody protein to the periplasmic space. There is no evidence that there are particular signal sequences which can direct the antibody protein to the medium; it appears rather that the outer membrane becomes leaky due to the mature antibody protein. Eukaryotic signal sequences often work successfully in bacteria, but the original heavy chain signal sequence of the antibody T15 failed in *E. coli* (SKERRA and PLÜCKTHUN 1991), probably because it cannot be cleaved properly due to its cysteine residue at the -1 position. Among bacterial signal sequences used successfully are those of the bacterial outer membrane protein A (*ompA*), alkaline phosphatase (*phoA*) and pectate lyase of *Erwinia carotovora* (*pelB*). The latter tolerates a sequence change, in which a very rare restriction site can be introduced, useful for PCR cloning (see below).

Beckwith and coworkers made the remarkable observation that a small amount of secretion to the periplasm of some proteins is detectable even if no signal sequence at all is used, especially in a *prlA* strain of *E. coli* (DERMAN et al. 1993). It remains to be investigated whether the observation of some amount of functional Fab, expressed without signal sequence in *E. coli* (CABILLY 1989), is related to this phenomenon.

For efficient secretion, the antibody gene must surely be fused precisely to a bacterial signal sequence. In the case of a scFv fragment, both domains making up the binding site are connected in a single protein (Figs. 3, 6, 7). In the case of Fv fragments and Fab fragments, however, both chains are unconnected. The most efficient way to guarantee coexpression and cose-

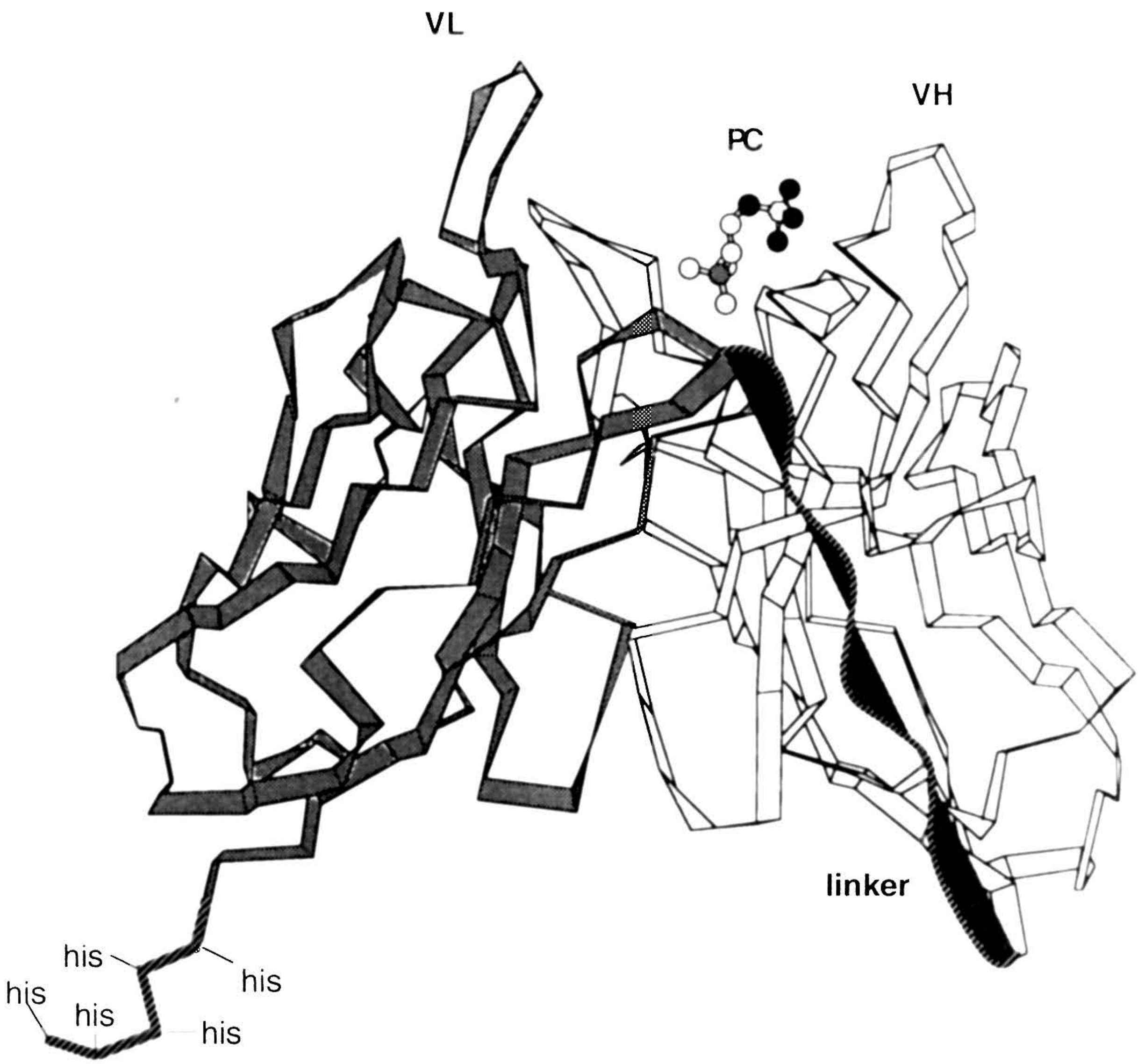


Fig. 7. A single chain Fv (scFv) fragment containing a histidine tail for purification with immobilized metal affinity chromatography. The linker is drawn very schematically, as it is now known to have no defined structure. (For details, see FREUND et al. 1993; see text)

cretion of both chains has been to design an aritificial operon, in which both genes, each fused to its signal sequence, are encoded in tandem on a single mRNA (SKERRA and PLÜCKTHUN 1988; BETTER et al. 1988) (Fig. 6). This way, simultaneous folding of heavy and light chains can occur in the periplasm. There is no indication that there is a higher kinetic barrier to association of V_H and V_L in an unlinked Fv fragment than in a scFv fragment, as evidenced by the similar expression yields (GLOCKSHUBER et al. 1990a). However, at equilibrium, some Fv fragments show significant dissociation into V_H and V_L which is of course concentration dependent (see below).

For protein production, the original dicistronic approach (Fig. 6) (SKERRA and PLÜCKTHUN 1988; BETTER et al. 1988) still seems to be the most ad-

vantageous, as only one promoter needs to be tightly regulated and only one plasmid needs to be maintained at high copy number. Nevertheless, other approaches have now been used as well. A two-promoter system has been used in a phage vector (BARBAS et al. 1991), as have been two-plasmid based systems (COLLET et al. 1992). These vector systems may add versatility to combinatorial libraries, but they will also add complications in stable production systems.

The mRNA ends at a transcription terminator, and there is evidence that efficient termination also protects the mRNA against exonucleolytic degradation (BELASCO and HIGGINS 1988 and references therein). Other elements of the plasmid include an antibiotic resistance, which, despite being a standard procedure, is worth a comment. The periplasmic leakiness induced by the antibody can lead to massive amounts of β-lactamase in the medium, degrading the antibiotic and making it possible for plasmid-free cells to grow (PLÜCKTHUN and SKERRA 1989). It is therefore better to resort to other antibiotics in prolonged growth experiments and fermentation, such as kanamycin or tetracycline. The origin of the expression plasmids based on the pUC series will give a high copy number (YANISCH-PERRON et al. 1985), but only at high temperature, and at room temperature, it falls below pBR322 (LIN-CHAO et al. 1992). Both for mutagenesis purposes and phage display (see below) an origin for a filamentous phage on the plasmid is useful (Fig. 6).

VII. Fermentation

Two reasons make it worthwhile to discuss the fermentation of bacteria producing antibodies in this context. First, the most efficient method to increase the amount of folded antibody per volume is by producing more cells, if the amount produced per cell is limited. Second, the fermenter allows for more careful control of the growth conditions. This is not only crucial for reproducibility, but also helps in understanding the physiology of the process.

An unexpected observation was the lack of periplasmic leakiness under fed-batch fermentation conditions (PACK et al. 1993). One possible explanation might be the artificially slowed growth in the fed-batch process, which might lead to a higher degree of cross-linking of the peptidoglycan cell wall (PARK 1987) and thus to a greater stability of the cell. Alternatively, the defined medium may be lacking a leakiness-inducing component. It was found to be crucial to use tightly regulated promoters (CARTER et al. 1992; BETTER et al. 1993; PACK et al. 1993), since otherwise plasmid loss is observed during cultivation. Before induction, nitrogen levels stay constant, indicating a metabolic balance of the repressed cells. After induction, this balance is lost, demonstrating the stress that antibody secretion constitutes for the bacterial cell. This stress appears to be dependent on the particular antibody sequence (KNAPPIK and PLÜCKTHUN, unpublished).

Fermentation now allows the production of functional antibody fragments (depending on the fragment and its sequence) with yields between 100 mg and 1 g per liter *E. coli* culture (CARTER et al. 1992; BETTER et al. 1993; PACK et al. 1993), demonstrating the general utility of the bacterial secretion technology.

VIII. Cloning Antibodies by Polymerase Chain Reaction

The availability of a large number of antibody sequences obtained by conventional cloning and the advent of PCR made it possible to define consensus primers to amplify antibody sequences (reviewed in LARRICK et al. 1992). PCR amplification of mRNA after reverse transcription and the amplification of rearranged V-D-J heavy chain or V-J light chain genes have been described (reviewed in LARRICK et al. 1992; HOOGENBOOM et al. 1992).

The "COOH-terminal" primers (reading toward the 5′ end of the RNA)[1] are not a problem: all constant domain sequences of mice and humans are known, and precisely matching primers can be made to amplify mRNA. Together with appropriate "NH_2-terminal" primers (reading towards the 3′ end of the mRNA)[1] sequences encoding the Fab fragment can be amplified. Alternatively, primers located in the J region (also completely known) can be used as the COOH-terminal primers. To amplify genomic V genes, different COOH-terminal primers have to be used for hybridizing at the end of the V genes (TOMLINSON et al. 1992). The NH_2-terminal primer can be located at the very beginning of the mature region of the V gene (ORLANDI et al. 1989; SASTRY et al. 1989). Alternatively, primers hybridizing to the signal sequence have been used successfully (JONES and BENDIG 1991) for amplifying mRNA. While the latter strategy conserves the NH_2-terminus of the mature gene, it requires an additional PCR step to introduce the gene into the bacterial expression vector and has been used mostly with eukaryotic expression vectors, and is less suitable for library construction.

The former method of PCR amplification may change the identity of the amino acids at the very beginning of the gene, since one of the primer-encoded sequences will be obtained by necessity. One may, however, convert these residues to any consensus sequence or to an experimentally determined protein sequence in a second round of PCR amplification or site-directed mutagenesis.

Most conveniently, the PCR product is cloned directly into the expression vector or phage display vector. This may be achieved by directly extending the PCR primers to include an "overhang" encoding a restriction site plus some extra bases to ensure cutting of the restriction enzyme close

[1] Winter and coworkers (see, e.g., HOOGENBOOM et al. 1992) have used a different nomenclature, in that the primer reading toward the 5′ end of the mRNA is called "forward," whereas the one priming synthesis toward the 3′ end is called "back."

to the end of the PCR fragment. Alternatively, a short precise primer can be used first to amplify the eukaryotic DNA or cDNA to eliminate any false priming by the overhang; a second round of amplification is then used to introduce the restriction site.

A variety of methods for introducing the PCR products into the vector, e.g. a bacterial secretion vector (Fig. 6), have been used. Many variations on this theme are possible, depending on whether bacterial secretion vectors, vectors for inclusion body formation, eukaryotic cDNA based vectors or eukaryotic genomic based vectors are to be used for receiving the PCR products.

IX. Purification

Purification of whole antibodies has usually relied on classical chromatography, antigen affinity chromatography or affinity chromatography using bacterial immunoglobulin-binding proteins such as staphylococcal proteins A, B, G or L (for summaries see BOYLE 1990; BOYLE and REIS 1987; FAULMANN et al. 1991; NILSON et al. 1992). However, the usefulness of this strategy for Fv or scFv fragments is fairly limited, as the bacterial proteins bind mostly to constant domains and only a few subgroups of V domains are recognized (INGANÄS et al. 1980; NILSON et al. 1992).

However, using affinity tails, any fragment can now be purified by rather convenient and reproducible procedures, and this technology can be carried out on a very large scale. The most convenient strategy is probably the use of a stretch of histidines at the COOH-terminus (SKERRA et al. 1991; LINDNER et al. 1992) (Fig. 7). This has been successfully tested with a scFv fragment of the form V_H-linker-V_L-His-5, with a V_L domain (V_L-His-5) and also with an Fab fragment, in which the His-5 tail was fused to C_H1. Since the heavy chain is practicably insoluble if not paired with a light chain, this amounts to a purification of assembled Fab fragments (KNAPPIK et al., unpublished) even though the two chains are not covalently linked. In the mouse κ V_L domain, the last two amino acids, Arg-Ala (numbers 108 and 109 according to Kabat), were replaced by histidines and only three additional His residues had to be added to the end. X-ray crystallography showed that this had no influence on the structure of the V_L domain (LINDNER et al. 1992).

Recent developments with immobilized metal ion affinity chromatography (IMAC) now also include a convenient detection system: by combining the metal ligand nitrilotriacetic acid (NTA) with biotin, the His-5 or His-6 containing protein can be detected in Western blots using phosphatase-labeled avidin (HOCHULI and PIESECKI 1992). Furthermore, the tail can also be used for obtaining a pseudocovalent binding: using Co^{2+} as the metal bound to NTA, which is attached to a solid support, the protein can be adsorbed. A later oxidation of Co^{2+} to Co^{3+} makes it exchange-inert,

thereby effectively "covalently" binding the protein to a solid surface in a predetermined orientation with the binding site still intact (SMITH et al. 1992).

A whole number of different affinity tags have been suggested for detection and purification, such as an epitope of the myc protein (EVAN et al. 1985; MUNRO and PELHAM 1986; WARD et al. 1989), a Ca^{2+}-dependent epitope useful both at the NH_2-terminal and the COOH-terminus (WELS et al. 1992a; PRICKETT et al. 1989; KNAPPIK and PLÜCKTHUN, unpublished), a peptide biotinylated in vivo in *E. coli* (MÜLLER and PLÜCKTHUN, unpublished; WEISS, personal communication), peptides binding to streptavidin (DEVLIN et al. 1990; SCHMIDT and SKERRA 1993), or epitopes encoded in the linker of a scFv fragment (BREITLING et al. 1991), but scale-up would be more costly than with the metal affinity procedure. Nevertheless, for detection purposes, these tags can be useful. For example, only three additional amino acids, Asp-Tyr-Lys, fused to the NH_2-terminal Asp residue of the V_L domain are sufficient to specifically detect the protein (KNAPPIK and PLÜCKTHUN, unpublished) with a specific antibody (Fig. 6). In the case of novel types of constructs or new antibody-like domains, it may also be useful to test the integrity of the protein with the simultaneous use of an NH_2-terminal and a COOH-terminal tag sequence, which has been shown to be possible with scFv fragments (GE et al., unpublished).

C. Expression of Antibody Fragments as Inclusion Bodies

The production of antibody proteins as cytoplasmic inclusion bodies in *E. coli* is also possible, and it does not differ greatly from the production of other recombinant proteins by this method. This was the strategy used in the first reports about expressing antibodies in *E. coli* (Boss et al. 1984; CABILLY et al. 1984). All types of antibody fragments (Fab, Fv, scFv and even the chains for the whole antibody) have since been produced this way (see, e.g., BOSS et al. 1984; CABILLY et al. 1984; WOOD et al. 1984; BIRD et al. 1988; HUSTON et al. 1988; FIELD et al. 1989; PANTOLIANO et al. 1991; CHEADLE et al. 1992; FREUND et al. 1993) and a variety of strains, plasmids and promoters have been used. There are no apparent requirements for the strain or expression system which would be specific for antibodies; any established production strain for *E. coli* inclusion bodies and most inducible strong promoters should be suitable. The use of the T7 system, as a particularly strong, but regulatable system, was found useful (see, e.g., HUSTON et al. 1991; FREUND et al. 1993). Fermentation of *E. coli* can also be carried out according to established principles (summarized by RIESENBERG 1991).

Too little is currently known to predict the ability of different sequences of form inclusion bodies (desired in this strategy) and their susceptibility to proteases. Recombinant proteins occasionally show signs of some degra-

dation, even when produced as inclusion bodies. The problem with antibodies is that there is considerable variability in the sequence and not all behavior observed for the antibodies tested so far may be generally valid. It is, for example, not yet known whether protease deficient strains are generally useful for the yield of inclusion bodies, and this may depend somewhat on the particular fragment and the particular sequence of the antibody of interest.

Usually, the inclusion body approach is carried out using genes not encoding signal sequences. Therefore, the antibody fragments stay in the cytoplasm and largely precipitate. Since precipitation is desired, it is useful to do exactly what needs to be avoided when secreting the antibody, namely, to grow the cells at higher temperature, e.g., 37°C. At lower temperature, some soluble Fab fragment has been observed which can be isolated from the cytoplasm (CABILLY 1989), but it has not been completely characterized in terms of the extent of its disulfide formation and stability.

Using secretion vectors, one may also isolate that portion of the secreted protein which precipitates after transport to the periplasm. This has been described for scFv fragments (COLCHER et al. 1990; GIBBS et al. 1991; WHITLOW and FILPULA 1991) and Fab fragments (SHIBUI et al. 1993). At higher temperatures (37°C), the protein still can be transported, but folding in the periplasm is often severely impaired, although apparently not for all antibodies (CARTER et al. 1992). Therefore, a heat inducible promoter is usually problematic for soluble expression and secretion, but ideal for inclusion body formation. The attraction of this at first paradoxical approach of refolding from periplasmic inclusion bodies comes from the fact that the periplasmic location protects the protein better from proteases. Therefore, some smaller antibody fragments may not reach the critical concentration required for precipitation because of competition from proteolysis in the cytoplasm. In the oxidizing milieu of the periplasm, some of the precipitated protein has disulfide linkages (PANTOLIANO et al. 1991), but it is not known what percentage of molecules has them and how many are correct. It is likely that a direct comparison of the yield from refolding periplasmic and cytoplasmic inclusion bodies depends on the exact vector constructions.

Cell growth, vector construction and inclusion body enrichment are straightforward (as there are no obvious specific differences from other recombinant proteins), but it should be noted that, if the protein is produced without a signal sequence, the 5′-coding region is derived from the mature eukaryotic protein, and not the prokaryotic signal sequence, and its mRNA secondary structure then plays a more important role. Consequently, the nucleotide sequence may have to be modified to avoid hairpin structures (WOOD et al. 1984). In one case, even additional amino acids had to be fused to the heavy chain of the Fab fragment to obtain good inclusion body formation (BUCHNER and RUDOLPH 1991). However, in other cases the inclusion body formation of a scFv fragment was found to be very efficient and straightforward with a T7 based expression system (see, e.g., HUSTON et

al. 1991; FREUND et al. 1993) but several other promoters have been used as well.

Several research groups have established refolding protocols. Fab fragments have been refolded at 10%–40% yield (see, e.g., BUCHNER and RUDOLPH 1991; SHIBUI et al. 1993), and scFv fragments have usually been refolded at 10%–20% yield (see, e.g., HUSTON et al. 1991; BUCHNER et al. 1992a,b; FREUND et al. 1993), in both cases with distinct variations due to the primary sequence. After refolding, the protein must be purified again and, especially, separated from incorrectly folded but perhaps soluble contaminating antibody protein (BUCHNER et al. 1992b). This is straightforward if an antigen affinity column is available, but it may require several steps of conventional chromatography if this is not available. It is thus not uncommon to obtain a yield of only a few percent of purified refolded protein (BUCHNER et al. 1992b) relative to the protein initially present in the inclusion body. In comparing the productiving of different *E. coli* strategies, it is crucial to keep this in mind.

What are the factors influencing the yield of in vitro refolding? Again, the refolding of antibodies is not principally different from that of other disulfide containing proteins (RUDOLPH 1990). First and foremost, the disulfide formation must be kinetically catalyzed and thermodynamically allowed. Using redox couples of reduced and oxidized glutathiones, concentrations of 1–2 m*M* reduced and 0.1–0.2 m*M* oxidized glutathione have been found useful (see, e.g., RUDOLPH 1990; BUCHNER and RUDOLPH 1991; HUSTON et al. 1991; BUCHNER et al. 1992a,b; FREUND et al. 1993), but the optimum may depend somewhat on the particular antibody (HUSTON et al. 1991). These conditions thermodynamically allow for formation of disulfide bonds, even if they appear at first sight to be reducing conditions, since the redox equilibrium of the protein disulfide bonds depends on the free energy of the folded protein, which stabilizes the oxidized form with respect to free cystine. Because of the importance of disulfide formation, it is useful to carry out refolding at high pH in order to speed up the disulfide reactions, since the reactive species is the thiolate anion. The aggregation of folding intermediates is a severe problem and probably the single most important side reaction lowering the yield in vitro and in vivo. Thus, rather low protein concentrations have to be used, but the unfolded protein may be added to the refolding mix in small portions since the folded protein has a much higher solubility. Additionally, additives such as 1 *M* arginine are often found useful, as they appear to increase the solubility of intermediates (RUDOLPH 1990). Too low a protein concentration may lead to gigantic volumes and prevents chain association in heterodimeric Fab fragments. Usually, refolding concentrations of 0.1–5 mg/ml are found useful (see, e.g., BUCHNER and RUDOLPH 1991; HUSTON et al. 1991, 1993; BUCHNER et al. 1992a,b; FREUND et al. 1993).

The addition of molecular chaperones in vitro has been investigated (BUCHNER et al. 1992a), yet without dramatic effects, just as in vivo (KNAPPIK

et al. 1993). Only very slight improvements in yield are seen, but the effort of providing stoichiometric amounts of such proteins makes this approach daunting on a technical scale.

All in all, in vitro refolding is a feasible strategy for a variety of antibody fragments. Yet, it is more laborious than production by secretion. Secretion is only now beginning to be optimized and, depending on the antibody primary sequence, already often levels of 1 g antibody per liter *E. coli* (CARTER et al. 1992) can be achieved. Crucial sequence determinants are beginning to be defined (KNAPPIK et al., unpublished). Therefore, if quantities of folded protein similar to those found in inclusion bodies can be obtained by secretion directly, it will always be the method of choice. If one particular antibody fragment needs to be produced routinely, however, optimizing a refolding/purification scheme can be an attractive option. Additionally, special applications such as the production of isotope-labeled proteins, as in NMR studies (FREUND et al. 1993), may make use of in vitro refolding because of the considerable expense of the label.

D. Antibody Fragments

Much of the preceding discussion on expression focused on protein folding in vivo and in vitro. A recurring theme was to make the molecule smaller, in the hope of increasing the yield of folding both in vitro and in vivo. It is necessary, however, to clearly understand the implications of working with small fragments of the antibody. Generally, working with smaller fragments can be very advantageous (see below) for many applications, and certain stability issues can now be overcome.

One may first ask: why make the antibody smaller at all. In most applications, binding the antigen is the central goal, and making the protein smaller simply removes much competing protein surface leading to non-specific reactions. In clinical applications, pharmacokinetics are an important issue. For instance, in tumor imaging experiments it is useful if the background clears rapidly, and clearance is dependent on the molecular size (COLCHER et al. 1990; YOKODA et al. 1992). Furthermore, smaller molecules penetrate tumor tissue much more efficiently (YOKODA et al. 1992). It is possible that smaller antibody fragments are per se less immunogenic, but as of yet there have been no quantitative investigations. The issue of antibody antigenicity has been summarized by ADAIR (1992). Perhaps most attractive, however, is the convenient accessibility of these small molecules by bacterial expression technology.

All domains of the antibody have a function, of course, but in applying antibodies in research, technology and medicine, binding the antigen is frequently the only function used. All antigen contacting regions are within V_H and V_L, and we must therefore also discuss which properties of the antibody might be lost if fragments consisting just of V_H and V_L are used. For instance, the constant domains of the Fab fragment contribute to stability in preventing the dissociation of V_H and V_L. However, V_H and V_L

can be linked covalently by a variety of methods (GLOCKSHUBER et al. 1990a), (see below). Many detection systems have relied on the constant domains, using either antibodies directed against the C regions or bacterial proteins which have an affinity for them (see Sect. B.IX). This problem can also be circumvented by using a "tag" sequence at the NH_2-terminus (WELS et al. 1992a), at the COOH-terminus (WARD et al. 1989) or in the linker of an scFv fragment (BREITLING et al. 1991). Alternatively, in ELISA applications, the detection enzymes (e.g., alkaline phosphatase) can be fused directly (WELS et al. 1992a), and in RIA applications, a metal binding domain can be fused to the antibody fragment (SAWYER et al. 1992).

The sugars of the C_H2 domains have often been used as a means of covalently linking the antibody to other chemicals, proteins or solid supports, since modifications there do not disturb the antigen binding activity. However, the same specific binding can be achieved with fragments not carrying C_H2 domains or any sugars, for instance, by encoding an additional cysteine at the end of the antibody fragment which can be selectively derivatized with maleimide or iodoacetyl derivatives (see, e.g., BERRY et al. 1991; BERRY and DAVIES 1992; CARTER et al. 1992; CUMBER et al. 1992; BERRY and PIERCE 1993; MCCARTNEY et al. 1993). This would be the only free cysteine in the molecule, all others being normally involved in disulfide bonds.

An additional feature of whole antibodies, which is lost in making fragment, is bivalency; however, even this feature can be restored with small fragments. Bivalent mini-antibodies (PACK and PLÜCKTHUN 1992; PACK et al. 1993) have been designed (see below) which self-assemble in *E. coli* and show the same avidity as a bivalent whole antibody, but have the size of only one Fab fragment.

There are nonetheless antibody functions which are lost when not using whole antibodies and for which no bacterial solutions have been reported yet. The most important ones are the binding of the F_C part to the F_C receptor (in the case of IgG, the receptors FcγRI, RII, and RIII), causing antibody-dependent cellular cytotoxicity (ADCC), and the ability to bind the complement factor C1q, the crucial step for complement activation (reviewed in MORGAN and WEIGLE 1987; SEGAL 1990; SCHUMAKER and POON 1990; SHIN et al. 1992; MORRISON 1992). Both of these functions need antibody glycosylation, presumably for the structural integrity of the C_H2 region of the molecule, even if the sugars themselves are not directly involved in the contact to the Fc receptor or C1q (DUNCAN and WINTER 1988; TAO and MORRISON 1989; GILLIES and WESOLOWSKI 1990; LUND et al. 1990). It remains to be seen whether functional analogs, perhaps involving variable domains binding to the effector molecule, can be found for bacteria.

I. Fv Fragments

From the available 3-D structures of antibodies, it is now obvious that all contacts with the antigen are within the V_H and V_L domains. While the

proteolytic digestion of an antibody to make an Fab fragment is usually straightforward (WEIR 1986), the proteolytic preparation of Fv fragments is less so. GIVOL and coworkers (INBAR et al. 1972; HOCHMAN et al. 1973, 1976; GIVOL 1991) could show that the proteolytically obtained Fv fragments they investigated were functional. However, the cleavage sites are not particularly preferred, and only certain antibodies give rise to good Fv preparations (SHARON and GIVOL 1976; TAKAHASHI et al. 1991), while in other cases complete functionality was not obtainable (SEN and BEYCHOK 1986) or only qualitatively ascertained (KAKIMOTO and ONOUE 1974; LIN and PUTNAM 1978; RETH et al. 1979).

These preparative problems have been overcome by using recombinant technology, and Fv fragments (Fig. 3) have been functionally expressed in *E. coli* (SKERRA and PLÜCKTHUN 1988) and in myeloma cells (RIECHMANN et al. 1988). Since a number of recombinant Fv fragments have now been made (see, e.g., WARD et al. 1989; FIELD et al. 1990; GLOCKSHUBER et al. 1991; MCMANUS and RIECHMANN 1991; TAKAHASHI et al. 1991; CHEADLE et al. 1992; ANTHONY et al. 1992), more conclusions can be drawn about their properties.

Fv fragments appear to have a lower interaction energy of V_H and V_L than Fab fragments, which are held together by the constant domains C_H1 and C_L as well (BIGELOW et al. 1974; AZUMA et al. 1974, 1978; HOCHMAN et al. 1976; MAEDA et al. 1976; KLEIN et al. 1979; STEVENS et al. 1980; HORNE et al. 1982; GLOCKSHUBER et al. 1990a). Nevertheless, many Fv fragments are stable and a few have been studied in detail. A certain range of interaction energies between V_H and V_L would be expected, since the interface also includes complementarity determining region 3 (CDR3) and part of CDR1 (CHOTHIA et al. 1985). It is possible that some very unstable Fv fragments have not been reported in the literature.

In the case of the phosphorylcholine binding antibody McPC603, the V_H-V_L association constant was measured and found to be about $10^6 M^{-1}$ (GLOCKSHUBER et al. 1990a). Since the antigen makes contact to both V_H and V_L, it stabilizes this interaction. In binding studies, a low *apparent* antigen binding constant may result because of chain dissociation. If it is deconvoluted into the V_H-V_L association constant and the antigen binding constant (identical to that of the Fab fragment or the whole antibody), the experimental data can be reproduced (GLOCKSHUBER et al. 1990a). Other Fv fragments, e.g., of the lysozyme binding antibody D1.3 (WARD et al. 1989) or of the anti-digoxin antibody 26-10 (ANTHONY et al. 1992) may have a higher V_H-V_L association constant, although exact quantitative data are not available. Taken together, these results suggest that the V_H-V_L heterodimer faithfully reproduces the binding site of the whole antibody.

Nevertheless, to make this a more general approach, methods are available (GLOCKSHUBER et al. 1990a) to stabilize the V_H-V_L interaction in antibodies: (a) by chemical crosslinking, (b) disulfide bond engineering and (c) genetic linking by a peptide linker (Fig. 7), to create a so-called single chain

Fv fragment (BIRD et al. 1988; HUSTON et al. 1988). It may be pointed out again that the reason for linking the domains lies in thermodynamic stability, but not in facilitating assembly in vivo, as the two unlinked domains do associate.

II. Single Chain Fv Fragments

Both orientations, V_H-linker-V_L and V_L-linker-V_H, have been realized (summarized in HUSTON et al. 1993). In the antibody McPC603, both were compared and no significant difference in the free energy of folding was found. Also, both were expressed at about the same level in *E. coli*, indicating a similar partitioning between folding and aggregation (KNAPPIK et al. 1993). However, different expression levels between both orientations have been noted in another case yet without molecular cause the molecular cause being pinpointed (ANAND et al. 1991b). It is conceivable that the presence of the linker might interfere with binding of some antigens, if, e.g., the NH_2-terminal residues make crucial contacts. In this case, it may be useful to switch the genetic order of the domains to free the particular NH_2-terminus.

Interestingly, the equilibrium denaturation curve of the scFv fragment is consistent with a two-state system (PANTOLIANO et al. 1991; KNAPPIK et al. 1993). This requires either that there is some coupling energy between V_H and V_L, i.e., that a state in which only one of the two domains is unfolded is not a stable intermediate (although it almost certainly is a kinetic intermediate, see below). Alternatively, the free energy of folding of V_H and V_L may be accidentally similar. However, the free energy of folding of the scFv of McPC603 is about 4.7 kcal/mol, whereas that of the V_L domain of the same antibody is only about 3 kcal/mol (LUPAS et al., unpublished). This indicates that more probably the folding of both domains is coupled.

A wide variety of linkers for connecting V_L and V_H have been tested (summarized in HUSTON et al. 1993), and it appears that there is great tolerance, as the linker seems to be a very passive entity contributing rather little to thermodynamic stability. It only appears to be critical that the linker has a length of around 15 residues, and of course it is important in this context how the end of the variable domain is defined. It is crucial for stability that the complete domain is present, as defined by the 3-D structure. One of the most frequenty used linkers has the sequence $(Gly_4\text{-}Ser)_3$ (HUSTON et al. 1988, 1991, 1993).

Recently, NMR experiments have made it possible to define the structural properties of this linker in a scFv fragment (FREUND et al. 1993). This was possible by comparing the NMR spectrum of the Fv fragment to the scFv fragment of the same antibody. The spectra were essentially superimposable, except for the linker region. This shows that the linker has essentially no influence on the structure of the variable domains at all (Fig. 7). Since the Fv fragment was obtained by periplasmic secretion and

that the same structure is obtained in both cases. To confirm that the additional peaks were indeed due to the linker, the scFv was selectively labeled with ^{15}N-glycine and ^{15}N-glycine/^{15}N-serine, and the spectrum correctly integrated to the expected number of amino acids in the linker.

The identification of the linker residues thus allowed the following conclusions to be drawn: the chemical shift of the glycine and serine residues shows almost no spread and is very similar to these amino acids in water, indicating that the linker must be largely exposed to solvent. Furthermore, the T_2 relaxation times are significantly longer than comparable residues elsewhere in the protein, indicating that the linker must be very flexible (FREUND et al. 1993). The linker can therefore adopt to a wide variety of structures. There are very few crosspeaks with the rest of the protein, indicating that there are few if any persistent contacts of the linker with the rest of the protein.

A number of laboratories have noticed a tendency of the VH and VL domains of some scFv fragments to not only associate intramolecularly but also intermolecularly (GRIFFITHS et al. 1993; WHITLOW et al. 1994; DESPLANCQ et al. 1994; MEZES, personal communication). This appears to depend on the primary sequence and on the length of the linker: The multimerization decreases with increasing linker length (DESPLANCQ et al. 1994). Whether the multimers reequilibrate rapidly or slowly also appears to depend on the system under study. HOLLIGER et al. (1993) made use of this phenomenon to force dimer formation by decreasing linker length to zero, and to also force two different scFv to come together to form bispecific scFvs.

Because of the genetic simplicity of the molecular system, a large number of fusion proteins have now been made with scFv fragments, including fusions with domains of protein A (GANDECHA et al. 1992; TAI et al. 1990), toxins (CHAUDARY et al. 1989, 1990; BATRA et al. 1990, 1991; KREITMAN et al. 1990; SEETHARAM et al. 1991; BRINKMANN et al. 1991, 1992; BUCHNER et al. 1992a,b; WELS et al. 1992b; NICHOLLS et al. 1993), alkaline phosphatase (WELS et al. 1992; KOHL et al. 1991), maltose binding protein (BRÉGÉGÈRE and BEDOUELLE 1992), interleukin-2 (SAVAGE et al. 1993), DNAse, RNAse (SPOONER and EPENETOS, personal communication), the COOH-terminal domain of the *E. coli* carboxyl carrier protein (BCCP) which is biotinylated in vivo in *E. coli* (MÜLLER and PLÜCKTHUN, unpublished; WEISS, personal communication) and avidin (SPOONER and EPENETOS, personal communication). In the production of these fusion proteins, both native secretion and in vitro refolding have been used. Fusion proteins do not have to be limited to single chain antibodies, however, and with bacterial systems Fab fragments made in bacteria have been fusion partners as well (see, e.g., SAWYER et al. 1992), following the work of NEUBERGER et al. (1984). Due to their genetic simplicity (requiring only a single gene), scFv fragments have been the antibody fragments of choice to establish expression systems in other hosts such as *Bacillus subtilis* (WU et al. 1993), the yeast *Schizosaccharomyces pombe* (DAVIS et al. 1991) and

plants (OWEN et al. 1992, discussed elsewhere in this volume). Nevertheless, because of its convenience, *E. coli* has remained the strain of choice for producing scFv fragments. A complete list of the reported scFv fragments has been compiled by HUSTON et al. (1993).

III. Disulfide-Linked Fv Fragments

Another strategy for linking V_H and V_L has been to design an intermolecular disulfide bond (GLOCKSHUBER et al. 1990a). Initial experiments of this type concentrated on the antibody McPC603 as a model system. Using a purely geometric approach (PABO and SUCHANEK 1986), all positions of V_H and V_L were searched for the best root mean square fit of the main chain atoms of any candidate pair of amino acids with any disulfide bond taken from the database. Using the best root mean square fits and excluding pairs involving proline residues or residues involved in antigen binding, several candidate disulfide bonds were found for McPC603. Two of these were tested experimentally, L56-H106 and L55-H108 (sequential numbering). The Fv fragment can be obtained in a disulfide-linked form directly from the periplasm, and it shows an almost indistinguishable antigen binding constant. The periplasmic protein can be purified directly by antigen affinity chromatography, and the protein obtained by this procedure is covalently linked (GLOCKSHUBER et al. 1990a).

The disulfide-linked Fv fragment appears to be much more resistant to irreversible denaturation than the unlinked Fv fragment. The stabilization is also much more dramatic than for the scFv fragment and greater than found in a chemically cross-linked Fv fragment. This may be the most important argument for pursuing this strategy. It thus appears that covalent linking is necessary, but not sufficient, for stabilizing the protein against irreversible denaturation. Rather, the type of covalent linking is important.

Least effective is the single chain strategy. Apparently, a rather loose link is created which does not prevent the Fv fragment from aggregation and precipitation after heating, consistent with the structural results from NMR (see above). Somewhat more efficient is chemical cross-linking (GLOCKSHUBER et al. 1990a), but this is not as easily reproducible for different antibodies as the other methods because of the different surface residues which can be cross-linked, and it is probably not as suitable as a general method or on a large scale. Most effective is the disulfide bond strategy. Probably, the location of the disulfide bonds does not allow much reversible opening and closing of the Fv fragment and may thus prevent aggregation of the Fv fragment at higher temperatures.

While very effective and demonstrating the principles, the particular disulfide bonds initially investigated are not necessarily of general utility. They connect CDR3 of V_H and CDR2 of V_L yet without interfering with binding of the antigen phosphorylcholine (PC). Because of the enormous structural variability, modeling of CDR3 is fairly difficult (CHOTHIA et al. 1989), and for most antibodies a similar juxtaposition of residues would not

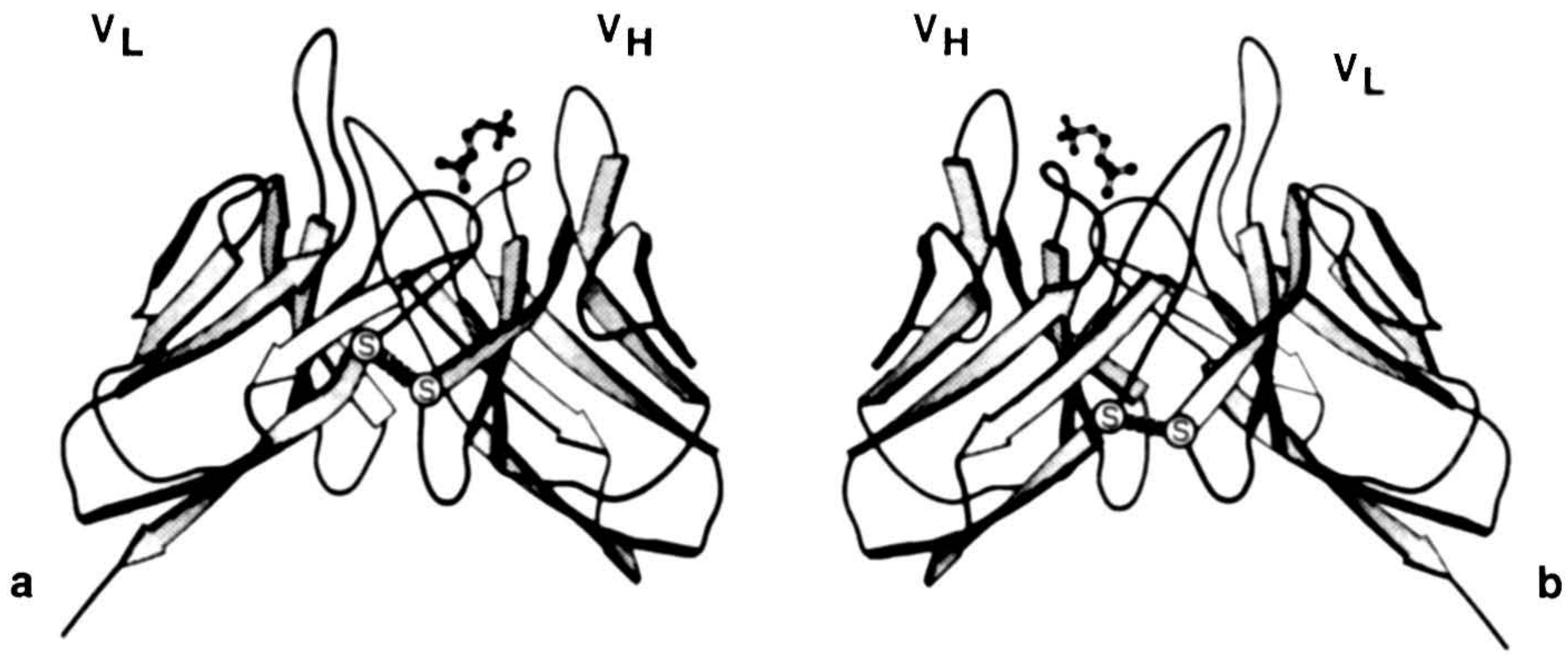
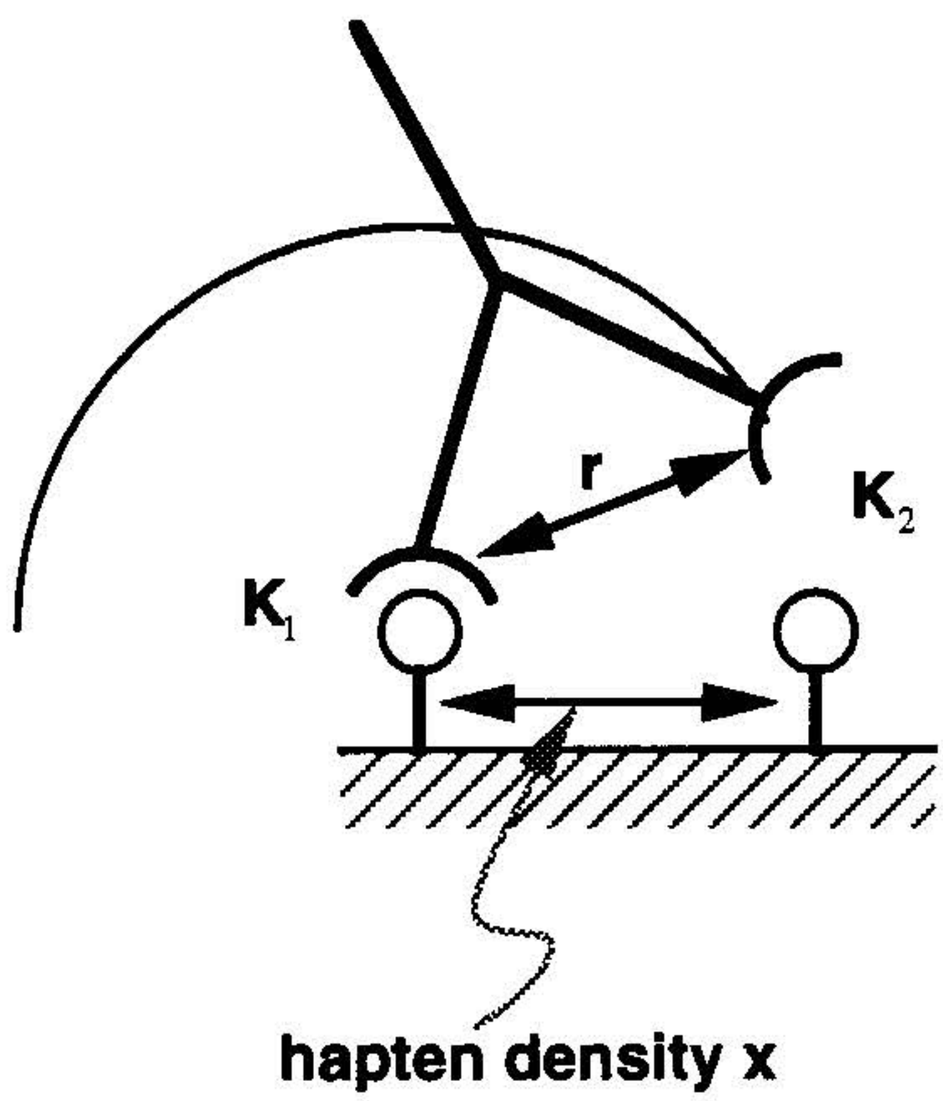


Fig. 8a,b. **a** Monoview of the Fv fragment of the antibody McPC603 with the positions labeled which were found suitable for linking V_H and V_L in the framework in a variety of Fv fragments. **b** Same fragment as in **a** rotated 180° about the y-axis. The similarity of the two possible positions, due to the molecular pseudo-twofold axis, is apparent. In each case, framework region 2 is connected to framework region 4. (For nomenclature see KABAT et al. 1991)

necessarily be expected. Therefore, a general solution to this problem was searched for (PLÜCKTHUN 1993; BRINKMANN et al. 1993; CARTER, personal communication). The Fv portions of ten different antibodies of known structure were superimposed, and all suitable positions for interchain disulfide bonds were calculated for all of them. Possible cross-links were superimposed, and two types of possible framework cross-links were identified for a majority of fragments (PLÜCKTHUN 1993) (Fig. 8). Because of the pseudo-twofold axis of the Fv fragment (due to the similarity of V_H and V_L), the two positions are structurally related by a rotation about this axis, and in both cases framework region 2 is linked to framework region 4 of the opposite chain (as defined in KABAT et al. 1991). None of the proposed positions will work well for all antibodies, and there is always some uncertainty about the exact geometry of the V_H-V_L interaction of a new antibody. Nevertheless, either of these positions appears to be reasonably promising (BRINKMANN et al. 1993) and need now to be tested on a sufficiently large number of different antibodies to evaluate their generality as a means of linking the component chains of an Fv fragment.

IV. Mini-antibodies

Nature has equipped antibodies with at least two binding sites. This way, they can bind to a surface (e.g., a bacterial surface or a virus particle) with higher functional affinity (sometimes called avidity) (CROTHERS and METZGER 1972; KARUSH 1976, 1978) (Fig. 9). Furthermore, a collection of different bivalent antibodies recognizing different epitopes on the same antigen can aggregate the antigen. This phenomenon will also be noticeable in solid phase binding assays such as ELISA.



$$K_1 = \frac{[Ab \cdot Ag]}{[Ab] \cdot [Ag]}$$

$$K_{obs} = 2 \cdot K_1 \cdot K_2$$

statistical factor

$$K_{obs} = 2 \cdot K_1 \cdot K_1 \cdot [Ab]_{eff} \cdot n_{haptens\ within\ reach}$$

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

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

Fig. 9. Enhancement of apparent equilibrium constant due to bivalency effects, according to the treatment of CROTHERS and METZGER (1972). K_1 is the observed binding constant for a monovalent fragment; K_{obs}, for a bivalent fragment. *[Ab], [Ag] and [Ab·Ag]* are the molar concentrations of antibody, antigen and complex, respectively (as if the number of molecules in the reaction well were evenly distributed). K_2 is the binding constant of the second binding site, made unitless by the constant effective antibody concentration $[Ab]_{eff}$, which results from constraining it within the hemisphere of radius r. The hapten density x is a two-dimensional concentration (molecules per area). N_{Avo} is Avogadro's number, used to convert molecular into molar concentrations

Why do dimeric or multimeric antibody molecules bind better and by how much? While the first part of the question is intuitively obvious, the second part is nontrivial. A number of quantitative approaches have been developed (summarized in KARUSH 1976, 1978), of which perhaps the most intuitive is that of CROTHERS and METZGER (1972). This says that the gain contributed by the second binding site is only observed if the antigen is on a surface or is polymeric. In this case, the gain is the product of the two binding constants of the two sites, (Fig. 9) the first being that which an Fab fragment would show, the second is the (dimensionless) constant of the second site, once the first site is bound. This is the binding at the average molar concentration of the second site constrained in the neighborhood of its epitope. The essence of the derivation is that the gain of having a second binding site should be proportional to the true intrinsic association constant K_1 and inversely proportional to the distance r of the two binding sites. Furthermore, it is proportional to the epitope density x on the surface. Therefore this gain is not a constant but dependent on many variables of a particular molecular system. This derivation neglects any energy needed to "bend" either antibody or antigen, complications from already occupied sites, surface layer effects (different ion concentrations, inaccessibility) and uneven microscopic distributions of the antigen on the surface. Neverthe-

less, it makes clear that a minimum distance is needed to have a limited chance of binding two different antigen molecules simultaneously.

While it is very desirable to access functional bivalent antibodies by bacterial technology, so far, no successful attempts to make functional whole antibodies in *E. coli* have been reported. Part of the problem is that, at least in IgG, for which a crystal structure is known (MARQUART et al. 1980; HARRIS et al. 1992), the two C_H2 domains make no protein-protein contact but their contact is entirely mediated by glycosylation (SUTTON and PHILLIPS 1983), which of course does not take place in *E. coli*. Therefore, other means of dimerization are required.

One may chemically link recombinant Fab fragments to $(Fab)_2$ fragments via the free cysteines. The most efficient and stable dimer is probably not obtained by forming a disulfide, but by using a *bis*-maleimide (BRENNAN et al. 1985). This can be done with recombinant Fab fragments produced in *E. coli* (see, e.g., CARTER et al. 1992). Similarly, scFv fragments can be linked this way (CUMBER et al. 1992).

It may be asked why disulfide-linked dimers do not form efficiently from proteins containing hinge peptides with cysteines in the periplasm in *E. coli* (CARTER et al. 1992; DE SUTTER et al. 1992) when formation of the intramolecular disulfide bond is possible. One reason may be that disulfide bonds do not cause dimer formation, they merely make covalent existing dimers which have previously formed by noncovalent forces. Intermolecular disulfide formation has been successfully obtained in *E. coli* between V_H and V_L (GLOCKSHUBER et al. 1990a), between C_H1 and C_L (BETTER et al. 1988; SKERRA and PLÜCKTHUN 1991) or between two coiled-coil helices (PACK and PLÜCKTHUN 1992) (see below). While equilibrium dimer formation has been seen in vitro with a model peptide of the hinge region (WÜNSCH et al. 1988), there may not be equilibrium conditions in the periplasm, and other proteins or peptides may instead be cross-linked to the hinge (DE SUTTER et al. 1992), or the structure may be trapped in nonnative intradomain disulfide bonds. Therefore, it appears crucial to provide a specific, noncovalent dimerization interface, in addition to any cysteine. Such examples will now be discussed.

1. Mini-antibodies Based on Coiled-Coil Helices

Methods have now been devised by which scFv fragments dimerize by themselves in vivo. These have been based on the tendency of amphipathic helices to dimerize or tetramerize. Two different principles have been exploited, that of antiparallel four-helix bundles and that of parallel coiled-coils (Figs. 10, 11).

Most useful is probably the attachment of these dimerization handles to scFv fragments (PACK and PLÜCKTHUN 1992), although in principle they can be added to Fab fragments (KOSTELNY et al. 1992) or Fv fragments. In the scFv fragment, unique heterodimers can be made, because wrong V_H-V_L pairing (which might occur during simultaneous in vivo expression or during

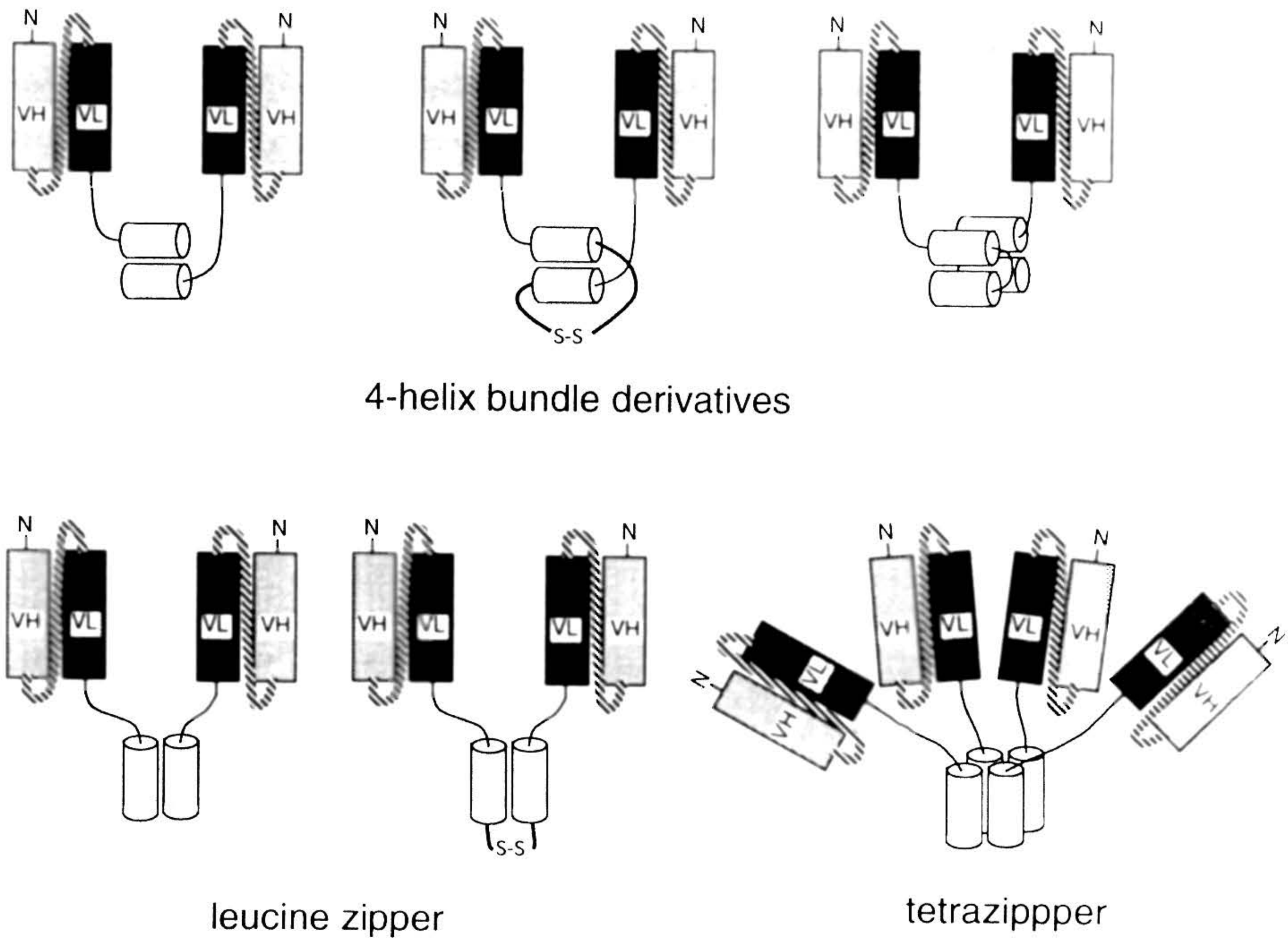


Fig. 10. Bivalent fragments which have been shown to assemble in *E. coli*. In each case, a single chain Fv (scFv) fragment is connected to a hinge region followed by an amphipathic helix. *Top row*, the helix comes from a 4-helix bundle design by deGrado and coworkers. *Top left*, only one helix is fused, but the predominant molecular species are dimers. *Top middle*, the helices are connected by a peptide which ends in a cysteine. *Top right*, two helices are fused in tandem and a 4-helix bundle is probably obtained, as very stable dimers are formed in vivo. *Bottom row*, a parallel coiled-coil helix from a leucine zipper is used. This design is suitable for making heterodimers. *Bottom right*, the sequence of the zipper is changed, as described in the text. (For details see PACK and PLÜCKTHUN 1992, PACK et al. 1993)

in vitro refolding) is not an issue. In these cases, the dimerization handle was not added to the scFv fragment directly, but rather separated by a hinge (the upper hinge from IgG3, known to be very flexible, as summarized by BURTON 1990). This way, an orientation and a distance between the two binding sites, similar to those in a whole antibody, are possible; this arrangement is also known to be very flexible (HARRIS et al. 1992).

Coiled-coil helices occur, for example, as dimerization devices in eukaryotic transcription factors (LANDSCHULZ et al. 1988). Because of their preference for leucine in every seventh position, they have been termed "leucine zippers." Leucine zippers have also been used as dimerization devices in other proteins (HU et al. 1990; BLONDEL and BEDOUELLE 1991). In a preliminary series of experiments, the zipper from the yeast transcrip-

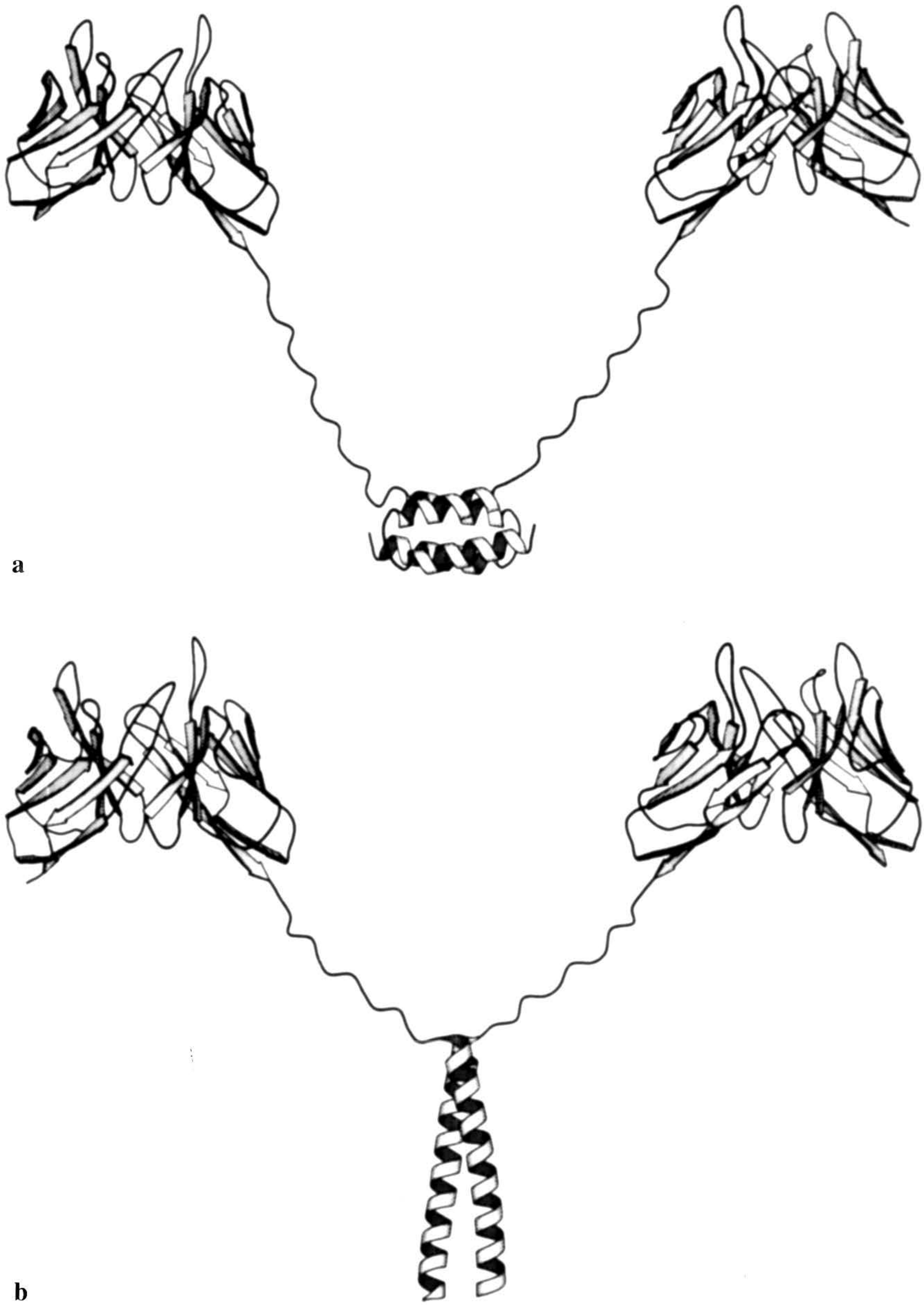


Fig. 11a,b. Molecular models of the dimeric mini-antibodies derived from the single chain Fv fragment of the mouse antibody McPC603 (SATOW et al. 1986). The hinge region was modeled according to a polyproline-II helix with $\phi = -78°$ and $\Psi = 149°$. **a** The 4-helix bundle design is shown, modeled on the *E. coli* protein Rop for packing. **b** The zipper design is shown, taken from the leucine zipper of the yeast transcription factor *GCN4*

tion factor GCN4 was used and shown to be suitable as a dimerization device, although it does not perform as well as four-helix bundles (see below). The reason for this difference is not quite clear, but it might have to do with the parallel arrangement of the helices, further constraining the two hinge regions. In the four-helix bundle, which is antiparallel, the length of the helix itself contributes to the distance the mini-antibody can span between two binding sites, and it may therefore increase the likelihood of two binding sites binding simultaneously to the surface.

Coiled-coil helices offer an approach to self-assembling heterodimers from *E. coli* eukaryotes (KOSTELNY et al. 1992). A number of different strategies have previously been employed to obtain heterodimeric antibodies or antibody fragments: (a) covalent cross-linking via disulfides or via heterobifunctional cross-linkers of whole antibodies (STAERZ et al. 1985; PEREZ et al. 1985) or monovalent fragments (NISONOFF and MANDY 1962; RASO and GRIFFIN 1981; BRENNAN et al. 1985), (b) forming two hybridomas to make a so-called heterohybridoma or quadroma (MILSTEIN and CUELLO 1983) or (c) cotransfection of a plasmid encoding the second antibody in a hybridoma producing the first (LENZ and WEIDLE 1990). All coexpression suffers from the statistical H-L pairing, in which the desired pairs may form only in small proportion, although preferential assumbly of the original H-L pairs is sometimes observed. Using scFv fragments and two complementary coiled-coil helices, such as from the transcription factors fos and jun, heterodimers can be made in vivo and the chain scrambling problem is greatly simplified. However, further alterations to the wild-type zipper sequences will be necessary to improve the yield of heterodimers (PACK and PLÜCKTHUN, unpublished).

It is now also possible to make tetrameric mini-antibodies. In this case, advantage was taken of the analysis of point mutants of coiled-coil helices (ALBER and KIM, personal communication). In the coiled-coil helix, repetitive heptads are found, with distinct preferences for hydrophobic β-branched amino acids in position a and d of the heptad (lettered from a to g) (COHEN and PARRY 1990; O'SHEA et al. 1989, 1991). If the naturally occurring Val (position a) and Leu (position d) are replaced with Leu and Ile, the molecule responds by tetramerizing (KIM and ALBER, personal communication). Again, this "tetrazipper" was linked to the scFv fragment by a hinge region, and gel filtration chromatography showed the tetrameric nature of these molecules, and an increase in binding avidity over dimers (PACK and PLÜCKTHUN, unpublished). Since the gain from multivalence is dependent on many molecular variables, it is very probable that there are cases in which this gain is large.

2. Mini-antibodies Based on Four-Helix Bundles

Four-helix bundles are compact folding motifs of natural proteins. Eisenberg, deGrado and coworkers described a synthetic four helix bundle (EISENBERG et al. 1986) made from either four single helices, two helix-turn-helix

peptides or one continuous chain (Ho and DEGRADO 1987; REGAN and DEGRADO 1988; HILL et al. 1990). In this design, all four-helices have the same sequence, and a surprisingly high thermodynamic stability has been measured (Ho and DEGRADO 1987).

Using this association principle, three different versions have been tested with scFv fragments. In the first, only one helix was fused to the scFv fragment, linked by the upper hinge region from mouse IgG3 (Figs. 10, 11). No tetramers are formed, however, but rather a distribution between monomers and dimers is obtained, presumably because the association energy of the helices is too weak. This can be improved by extending the helix with a hydrophilic peptide ending in a cysteine (PACK and PLÜCKTHUN 1992). The covalently linked scFv mini-antibody is stable and remains in the dimer state. In this case, the peptide linker may be in the way of tetramer association.

Most stable, however, is the construct carrying a helix-turn-helix motif (Figs. 10, 11). In this case, very little degradation is observed and avidities are obtained identical to whole IgA (PACK et al. 1993). It appears that these amphipathic helices are compatible with transport through the bacterial membrane and cause no problems in folding of the scFv fragments.

E. Conclusions

Producing and characterizing an engineered antibody fragment is the prerequisite for improving its performance, no matter what the application. Since all cloning and mutagenesis is carried out in *E. coli*, it is convenient to use these bacteria for expression as well. It is possible to produce surprisingly complex multisubunit structures in *E. coli* with correct folding and assembly, provided the expression strategy is adapted to the physiology of the growing cell. In this case not only can small amounts of the antibody constructs be obtained rapidly for laboratory testing, but amounts useful for clinical and industrial applications can now be produced by fermentation. Combined with library selection and current developments in imitating affinity maturation, bacterial antibody technology will become an integral part of any research involving immunoglobulins.

References

Abel CA, Grey HM (1968) Studies on the structure of mouse γA-myeloma proteins. Biochemistry 7:2682–2688

Adair JR (1992) Engineering antibodies for therapy. Immunol Rev 130:5–40

Anand NN, Dubuc G, Phipps J, MacKenzie CR, Sadowska J, Young NM, Bundle DR, Narang SA (1991a) Synthesis and expression in *Escherichia coli* of cistronic DNA encoding an antibody fragment specific for a *Salmonella* serotype B O-antigen. Gene 100:39–44

Anand NN, Mandal S, MacKenzie CR, Sadowska J, Sigurskjold B, Young NM, Bundle DR, Narang SA (1991b) Bacterial expression and secretion of various

single-chain Fv genes encoding proteins specific for a *Salmonella* serotype B O-antigen. J Biol Chem 266:21874–21879

Anthony J, Near R, Wong SL, Iida E, Ernst E, Wittekind M, Haber E, Ng SC (1992) Production of stable anti-digoxin-Fv in *Escherichia coli*. Mol Immunol 29:1237–1247

Azuma T, Hamaguchi K, Migita S (1974) Interactions between immunoglobulin polypeptide chains. J Biochem 76:685–693

Azuma T, Kobayashi O, Goto Y, Hamaguchi K (1978) Monomer-dimer equilibria of a Bence Jones protein and its variable fragment. J Biochem 83:1485–1492

Barbas CF III, Kang AS, Lerner RA, Benkovic SJ (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci USA 88:7978–7982

Barbas CF III, Bain JD, Hoekstra DM, Lerner RA (1992a) Semisynthetic combinatorial antibody libraries: A chemical solution to the diversity problem. Proc Natl Acad Sci USA 89:4457–4461

Barbas CF III, Björling E, Chiodi F, Dunlop N, Cababa D, Jones TM, Zebedee SL, Persson MAA, Nara PL, Norrby E, Burton DR (1992b) Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus in vitro. Proc Natl Acad Sci USA 89:9339–9343

Bardwell JCA, McGovern K, Beckwith J (1991) Identification of a protein required for disulfide bond formation in vivo. Cell 67:581–589

Bardwell JCA, Lee JO, Jander G, Martin N, Belin D, Beckwith J (1993) A pathway for disulfide bond formation in vivo. Proc Natl Acad Sci USA 90:1038–1042

Batra JK, FitzGerald D, Gately M, Chaudhary VK, Pastan I (1990) Anti-Tac(Fv)-PE40, a single chain antibody *Pseudomonas* fusion protein directed at interleukin 2 receptor bearing cells. J Biol Chem 265:15198–15202

Batra JK, FitzGerald DJ, Chaudhary VK, Pastan I (1991) Single-chain immunotoxins directed at the human transferrin receptor containing *Pseudomonas* exotoxin A or diphtheria toxin: anti-TFR(Fv)-PE40 and DT388-anti-TFR(Fv). Mol Cell Biol 11:2200–2205

Beckwith JR, Zipser D (eds) (1970) The lactose operon. Cold Spring Harbor Laboratory, Cold Spring Harbor

Belasco JG, Higgins CF (1988) Mechanisms of mRNA decay in bacteria: a perspective. Gene 72:15–23

Bergmann LW, Kuehl WM (1979) Formation of an intrachain disulfide bond on nascent immunoglobulin light chains. J Biol Chem 254:8869–8876

Berry MJ, Davies J (1992) Use of antibody fragments in immunoaffinity chromatography: comparison of Fv fragments, VH fragments and paralog peptides. J Chromatogr 597:239–245

Berry MJ, Pierce JJ (1993) Stability of immunoadsorbents comprising antibody fragments – comparison of Fv fragments and single-Chain Fv fragments. J Chromatogr 629:161–168

Berry MJ, Davies J, Smith CG, Smith I (1991) Immobilization of Fv antibody fragments on porous silica and their utility in affinity chromatography. J Chromatogr 587:161–169

Better M, Chang CP, Robinson RR, Horwitz AH (1988) *Escherichia coli* secretion of an active chimeric antibody fragment. Science 240:1041–1043

Better M, Bernhard SL, Lei SP, Fishwild DM, Lane JA, Carroll SF, Horwitz AH (1993) Potent anti-Cd5 ricin-A chain immunoconjugates from bacterially produced Fab′ and Fab'_2. Proc Natl Acad Sci USA 90:457–461

Bigelow CC, Smith BR, Dorrington KJ (1974) Equilibrium and kinetic aspects of subunit association in immunoglobulin G. Biochemistry 13:4602–4608

Bird RE, Hardman KD, Jacobson JW, Johnson S, Kaufman BM, Lee S-M, Lee T, Pope SH, Riordan GS, Whitlow M (1988) Single-chain antigen-binding proteins. Science 242:423–426

Blondel A, Bedouelle H (1991) Engineering the quaternary structure of an exported protein with a leucine zipper. Protein Eng 4:457–461

Boss MA, Kenten JH, Wood CR, Emtage JS (1984) Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in *E. coli*. Nucleic Acids Res 12:3791–3806

Boyle MDP (ed) (1990) Immunoglobulin binding proteins, vol 1. Academic Press, San Diego

Boyle MDP, Reis KJ (1987) Bacterial Fc receptors. Biotechnology 5:697–703

Brandts JF, Halvorson HR, Brennan M (1975) Consideration of the possibility that the slow step in protein denaturation reactions is due to cis-trans isomerism of proline residues. Biochemistry 14:4953–4963

Brégégère F, Bedouelle H (1992) Expression, exportation et purification des fragments d'anticorps fusionnés à la protéine affine du maltose d'*Escherichia coli*. C R Acad Sci [III] 314:527–532

Breitling F, Dübel S, Seehaus T, Klewinghaus I, Little M (1991) A surface expression vector for antibody screening. Gene 104:147–153

Brennan M, Davison PF, Paulus H (1985) Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G_1 fragments. Science 229:81–83

Brinkmann U, Pai LH, FitzGerald DJ, Willingham M, Pastan I (1991) B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. Proc Natl Acad Sci USA 88:8616–8620

Brinkmann U, Buchner J, Pastan I (1992) Independent domain folding of *Pseudomonas* exotoxin and single-chain immunotoxins: influence of interdomain connections. Proc Natl Acad Sci USA 89:3075–3079

Brinkmann U, Reiter Y, Jung SH, Lee B, Pastan I (1993) A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. Proc Natl Acad Sci USA 90:7538–7542

Buchner J, Rudolph R (1991) Renaturation, purification and characterization of recombinant Fab-fragments produced in *Escherichia coli*. Biotechnology 9: 157–162

Buchner J, Brinkmann U, Pastan I (1992a) Renaturation of a single-chain immunotoxin facilitated by chaperones and protein disulfide isomerase. Biotechnology 10:682–685

Buchner J, Pastan I, Brinkmann U (1992b) A method for increasing the yield of properly folded recombinant fusion proteins: single-chain immunotoxins from renaturation of bacterial inclusion bodies. Anal Biochem 205:263–270

Burton DR (1990) In: Metzger H (ed) Fc receptors and the action of antibodies. American Society of Microbiology, Washington, pp 31–54

Cabilly S (1989) Growth at sub-optimal temperatures allows the production of functional, antigen-binding Fab fragments in *Escherichia coli*. Gene 85:553–557

Cabilly S, Riggs AD, Pande H, Shively JE, Holmes WE, Rey M, Perry LJ, Wetzel R, Heyneker HL (1984) Generation of antibody activity from immunoglobulin polypeptide chains produced in *Escherichia coli*. Proc Natl Acad Sci USA 81:3273–3277

Carter P, Kelley RF, Rodrigues ML, Snedecor B, Covarrubias M, Velligan MD, Wong WLT, Rowland AM, Kotts CE, Carver ME, Yang M, Bourell JH, Shepard HM, Henner D (1992) High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. Biotechnology 10: 163–167

Chang CN, Landolfi NF, Queen C (1991) Expression of antibody Fab domains on bacteriophage surfaces. Potential use for antibody selection. J Immunol 147: 3610–3614

Chaudhary VK, Queen C, Junghans RP, Waldmann TA, FitzGerald DJ, Pastan I (1989) A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas exotoxin*. Nature 339:394–397

Chaudhary VK, Gallo MG, FitzGerald DJ, Pastan I (1990) A recombinant single-chain immunotoxin composed of anti-Tac variable regions and a truncated diphtheria toxin. Proc Natl Acad Sci USA 87:9491–9494

Cheadle C, Hook LE, Givol D, Ricca GA (1992) Cloning and expression of the variable regions of mouse myeloma protein MOPC315 in *E. coli*: recovery of active Fv fragments. Mol Immunol 29:21–30

Chothia C, Novotny J, Bruccoleri R, Karplus M (1985) Domain association in immunoglobulin molecules. The packing of variable domains. J Mol Biol 186: 651–663

Chothia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill SJ, Air G, Sheriff S, Padlan EA, Davies D, Tulip WR, Colman PM, Spinelli S, Alzari PM, Poljak RJ (1989) Conformations of immunoglobulin hypervariable regions. Nature 342: 877–883

Clackson T, Hoogenboom HR, Griffiths AD, Winter G (1991) Making antibody fragments using phage display libraries. Nature 352:624–628

Cockle SA, Young NM (1985) The thiol groups of mouse immunoglobulin A. Incomplete formation of the Cα1-domain disulphide bridge. Biochem J 225: 113–125

Cohen C, Parry DAD (1990) α-Helical coiled coils and bundles: how to design an α-helical protein. Proteins 7:1–15

Colcher D, Bird R, Roselli M, Hardman KD, Johnson S, Pope S, Dodd SW, Pantoliano MW, Milenic DE, Schlom J (1990) In vivo tumor targeting of a recombinant single-chain antigen-binding protein. J Natl Cancer Inst 82: 1191–1197

Collet TA, Roben P, O'Kennedy R, Barbas CF, Burton DR, Lerner RA (1992) A binary plasmid system for shuffling combinatorial antibody libraries. Proc Natl Acad Sci USA 89:10026–10030

Creighton TE (1978) Experimental studies of protein folding and unfolding. Prog Biophys Mol Biol 33:231–297

Crothers DM, Metzger H (1972) The influence of polyvalency on the binding properties of antibodies. Immunochemistry 9:341–357

Cumber AJ, Ward ES, Winter G, Parnell GD, Wawrzynczak EJ (1992) Comparative stabilities in vitro and in vivo of a recombinant mouse antibody FvCys fragment and a bisFvCys conjugate. J Immunol 149:120–126

Cwirla SE, Peters EA, Barrett RW, Dower WJ (1990) Peptides on phage: a vast library of peptides for identifying ligands. Proc Natl Acad Sci USA 87:6378–6382

Davis GT, Bedzyk WD, Voss EW, Jacobs TW (1991) Single-chain antibody (SCA) encoding genes: one-step construction and expression in eukaryotic cells. Biotechnology 9:165–169

De Sutter K, Remaut E, Fiers W (1992) Disulphide bridge formation in the periplasm of *Escherichia coli*: beta-lactamase:: human IgG3 hinge fusions as a model system. Mol Microbiol 6:2201–2208

Derman AI, Puziss JW, Bassford PJ, Beckwith J (1993) A signal sequence is not required for protein export in *prlA* mutants of *Escherichia coli*. EMBO J 12: 879–888

Desplancq D, King DJ, Lawson ADG, Mountain A (1994) Multimerisation behaviour of single-chain Fv variants for the tumour-binding antibody B72.3. Protein Eng, in press

Devlin JJ, Panganiban LC, Devlin PE (1990) Random peptide libraries: a source of specific protein binding molecules. Science 249:404–406

Duncan AR, Winter G (1988) The binding site for Clq on IgG. Nature 332:738–740

Ehretsmann CP, Carpousis AJ, Krisch HM (1992) mRNA degradation in prokaryotes. FASEB J 6:3186–3192

Eisenberg D, Wilcox W, Eshita SM, Pryciak PM, Ho SP, DeGrado WF (1986) The design, synthesis, and crystallization of an alpha-helical peptide. Proteins 1:16–22

Evan GI, Lewis GK, Ramsay G, Bishop JM (1985) Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. Mol Cell Biol 5: 3610–3616

Faulmann EL, Duvall JL, Boyle MDP (1991) Protein B: a versatile bacterial Fc-binding protein selective for human IgA. Biotechniques 10:748–755

Felici F, Castagnoli L, Musacchio A, Jappelli R, Cesareni G (1991) Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. J Mol Biol 222:301–310

Field H, Yarranton GT, Rees AR (1990) Expression of mouse immunoglobulin light and heavy chain variable regions in *Escherichia coli* and reconstitution of antigen-binding activity. Protein Eng 3:641–647

Fischer G, Schmid FX (1990) The mechanism of protein folding. Implications of in vitro refolding models for de novo protein folding and translocation in the cell. Biochemistry 29:2205–2212

Francisco JA, Earhart CF, Georgiou G (1992) Transport and anchoring of β-lactamase to the external surface of *Escherichia coli*. Proc Natl Acad Sci 89:2713–2717

Francisco JA, Stathopoulos C, Warren RAJ, Kilburn DG, Georgiou G (1993) Specific adhesion and hydrolysis of cellulose by intact *Escherichia coli* expressing surface anchored cellulase or cellulose binding domains. Biotechnology 11: 491–495

Freedman RB, Bulleid NJ, Hawkins HC, Paver JL (1989) Role of protein disulphide-isomerase in the expression of native proteins. Biochem Soc Symp 55:167–192

Freund C, Ross A, Guth B, Plückthun A, Holak T (1993) Characterization of the linker peptide of the single-chain Fv fragment of an antibody of NMR spectroscopy. FEBS Lett 320:97–100

Fuchs P, Breitling F, Dübel S, Seehaus T, Little M (1991) Targeting recombinant antibodies to the surface of *Escherichia coli*: fusion to a peptidoglycan associated lipoprotein. Biotechnology 9:1369–1372

Gandecha AR, Owen MRL, Cockburn B, Whitelam GC (1992) Production and secretion of a bifunctional staphylococcal protein A:: antiphytochrome single-chain Fv fusion protein in *Escherichia coli*. Gene 122:361–365

Garrard LJ, Yang M, O'Connell MP, Kelley RF, Henner DJ (1991) Fab assembly and enrichment in a monovalent phage display system. Biotechnology 9:1373–1377

Gibbs RA, Posner BA, Filpula DR, Dodd SW, Finkelman MAJ, Lee TK, Wroble M, Whitlow M, Benkovic SJ (1991) Construction and characterization of a single-chain catalytic antibody. Proc Natl Acad Sci USA 88:4001–4004

Gilbert HF (1990) Molecular and cellular aspects of thiol-disulfide exchange. Adv Enzymol 63:69–172

Gillies SD, Wesolowski JS (1990) Antigen binding and biological activities of engineered mutant chimeric antibodies with human tumor specificities. Hum Antibod Hybridomas 1:47–54

Givol D (1991) The minimal antigen-binding fragment of antibodies – Fv fragment. Mol Immunol 28:1379–1386

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

Glockshuber R, Steipe B, Huber R, Plückthun A (1990b) Crystallization and preliminary X-ray studies of the V_L domain of the antibody McPC603 produced in *Escherichia coli*. J Mol Biol 213:613–615

Glockshuber R, Stadlmüller J, Plückthun A (1991) Mapping and modification of an antibody hapten binding site: a site-directed mutagenesis study of McPC603. Biochemistry 30:3049–3054

Glockshuber R, Schmidt T, Plückthun A (1992) The disulfide bonds in antibody variable domains: effects on stability, folding in vitro, and functional expression in *Escherichia coli*. Biochemistry 31:1270–1279

Goldenberg DP (1992) Native and non-native intermediates in the BPTI folding pathway. Trends Biochem 17:257–261

Goto Y, Hamaguchi K (1979) The role of the intrachain disulfide bond in the conformation and stability of the constant fragment of the immunoglobulin light chain. J Biochem 86:1433–1441

Goto Y, Hamaguchi K (1982) Unfolding and refolding of the constant fragment of the immunoglobulin light chain. J Mol Biol 156:891–910

Goto Y, Hamaguchi K (1986) Conformation and stability of the constant fragment of the immunoglobulin light chain containing an intramolecular mercury bridge. Biochemistry 25:2821–2828

Goto Y, Ichimura N, Hamaguchi K (1988) Effects of ammonium sulfate on the unfolding and refolding of the variable and constant fragments of an immunoglobulin light chain. Biochemistry 27:1670–1677

Gram H, Marconi L-A, Barbas III CF, Collet TA, Lerner RA, Kang AS (1992) In vitro selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. Proc Natl Acad Sci USA 89:3576–3580

Greenwood J, Willis AE, Perham RN (1991) Multiple display of foreign peptides on a filamentous bacteriophage. Peptides from *Plasmodium falciparum* circumsporozoite protein as antigens. J Mol Biol 220:821–827

Griffiths AD, Malmquist M, Marks JD, Bye JD, Embleton MJ, McCafferty J, Baier M, Holliger KP, Gorick BD, Hughes-Jones NC, Hoogenboom HR, Winter G (1993) Human anti-self antibodies with high specificity from phage display libraries. EMBO J 12:725–734

Haber E (1964) Recovery of antigenic specificity after denaturation and complete reduction of disulfides in a papain fragment of antibody. Proc Natl Acad Sci USA 52:1099–1106

Harris LJ, Larson SB, Hasel KW, Day J, Greenwood A, McPherson A (1992) The three-dimensional structure of an intact monoclonal antibody for canine lymphoma. Nature 360:369–372

Hayano T, Takahashi N, Kato S, Maki N, Suzuki M (1991) Two distinct forms of peptidylprolyl-*cis-trans*-isomerase are expressed separately in periplasmic and cytoplasmic compartments of *Escherichia coli* cells. Biochemistry 30:3041–3048

Hill CP, Anderson DH, Wesson L, DeGrado WF, Eisenberg D (1990) Crystal structure of α_1: implications for protein design. Science 249:543–546

Ho SP, DeGrado WF (1987) Design of a 4-helix bundle protein: synthesis of peptides which self-associate into a helical protein. J Am Chem Soc 109:6751–6758

Hochman J, Inbar D, Givol D (1973) An active antibody fragment (Fv) composed of the variable portions of heavy and light chains. Biochemistry 12:1130–1135

Hochman J, Gavish M, Inbar D, Givol D (1976) Folding and interaction of subunits at the antibody combining site. Biochemistry 15:2706–2710

Hochuli E, Piesecki S (1992) Interaction of hexahistidine fusion proteins with nitrilotriacetic acid-chelated Ni^{2+} ions. Methods: A Companion to Methods Enzymol 4:68–72

Holland IB, Kenny B, Steipe B, Plückthun A (1990) Secretion of heterologous proteins in *Escherichia coli*. Methods Enzymol 182:132–143

Holliger P, Prospero T, Winter G (1993) "Diabodies": small bivalent and bispecific antibody fragments. Proc Natl Acad Sci USA 90:6444–6448

Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P, Winter G (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res 19:4133–4137

Hoogenboom HR, Marks JD, Griffiths AD, Winter G (1992) Building antibodies from their genes. Immunol Rev 130:41–68

Horne C, Klein M, Polidoulis I, Dorrington KJ (1982) Noncovalent association of heavy and light chains of human immunoglobulins. III. Specific interactions between V_H and V_L. J Immunol 129:660–664

Hu JC, O'Shea EK, Kim PS, Sauer RT (1990) Sequence requirements for coiled-coils: analysis with λ repressor-GCN4 leucine zipper fusions. Science 250:1400–1403

Huse WD, Sastry L, Iverson SA, Kang AS, Alting-Mees M, Burton DR, Benkovic SJ, Lerner RA (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. Science 246:1275–1281

Huston JS, Levinson D, Mudgett-Hunter M, Tai M-S, Novotny J, Margolies MN, Ridge RJ, Bruccoleri RE, Haber E, Crea R, Oppermann H (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. Proc Natl Acad Sci USA 85:5879–5883

Huston JS, Mudgett-Hunter M, Tai M-S, McCartney J, Warren F, Haber E, Oppermann H (1991) Protein engineering of single-chain Fv analogs and fusion proteins. Methods Enzymol 203:46–88

Huston JS, McCartney J, Tai MS, Mottola-Hartshorn C, Jin D, Warren F, Keck P, Oppermann H (1993) Medical applications of single-chain antibodies. Int Rev Immunol (in press)

Inbar D, Hochman J, Givol D (1972) Localization of antibody-combining sites within the variable portions of heavy and light chains. Proc Natl Acad Sci USA 69:2659–2662

Inganäs M, Johansson SGO, Bennich HH (1980) Interaction of human polyclonal IgE and IgG from different species with protein A from *Staphylococcus aureus*: demonstration of protein-A-reactive sites located in the Fab_2 fragment of human IgG. Scand J Immunol 12:23–31

Jaenicke R (1993) Role of accessory proteins in protein folding. Curr Opin Struct Biol 3:104–112

Jones ST, Bendig MM (1991) Rapid PCR-cloning of full-length mouse immunoglobulin variable regions. Biotechnology 9:88–89

Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C (1991) Sequences of proteins of immunological interest, 5th edn. National Institutes of Health, Bethesda

Kakimoto K, Onoue K (1974) Characterization of the Fv fragment isolated from a human immunoglobulin M. J Immunol 112:1373–1382

Kamitani S, Akiyama Y, Ito K (1992) Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. EMBO J 11:57–62

Kang AS, Barbas CF, Janda KD, Benkovic SJ, Lerner RA (1991) Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. Proc Natl Acad Sci USA 88:4363–4366

Karush F (1976) Multivalent binding and functional affinity. Contemp Top Mol Immunol 5:217–228

Karush F (1978) The affinity of antibody: range, variability and the role of multivalence. In: Litman GW, Good RA (eds) Immunoglobulins. Plenum Publishing, New York, pp 85–116

Klauser T, Pohlner J, Meyer TF (1992) Selective extracellular release of cholera toxin B subunit by *Escherichia coli*: dissection of Neisseria Igaβ-mediated outer membrane tranport. EMBO J 11:2327–2335

Klein M, Kortan C, Kells DIC, Dorrington KJ (1979) Equilibrium and kinetic aspects of the interaction of isolated variable and constant domains of light chain with the Fd′ fragment of immunoglobulin G. Biochemistry 18:1473–1481

Knappik A, Krebber C, Plückthun A (1993) The effect of folding catalysts on the in vivo folding process of different antibody fragments expressed in *Escherichia coli*. Biotechnology 11:77–83

Kohl J, Rüker F, Himmler G, Razazzi E, Katinger H (1991) Cloning and expression of an HIV-1 specific single-chain Fv region fused to *Escherichia coli* alkaline phosphatase. Ann NY Acad Sci 646:106–114

Kostelny SA, Cole MS, Tso JY (1992) Formation of a bispecific antibody by the use of leucine zippers. J Immunol 148:1547–1553

Kreitman RJ, Chaudhary VK, Waldmann T, Willingham MC, FitzGerald DJ, Pastan I (1990) The recombinant immunotoxin anti-Tac(Fv)-*Pseudomonas* exotoxin 40 is cytotoxic toward peripheral blood malignant cells from patients with adult T-cell leukemia. Proc Natl Acad Sci USA 87:8291–8295

Landschulz WH, Johnson PF, McKnight SL (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science 240:1759–1764

Lang K, Schmid FX (1988) Protein-disulphide isomerase and prolyl isomerase act differently and independently as catalysts of protein folding. Nature 331:453–455

Larrick JW, Wallace EF, Coloma MJ, Bruderer U, Lang AB, Fry KE (1992) Therapeutic human antibodies derived from PCR amplification of B-cell variable regions. Immunol Rev 130:69–85

Lenz H, Weidle UH (1990) Expression of heterobispecific antibodies by genes transfected into producer hybridoma cells. Gene 87:213–218

Lin-Chao S, Chen WT, Wong TT (1992) High copy number of the pUC plasmids results from a Rom/Rop-suppressible point mutation in RNAII. Mol Microbiol 6:3385–3390

Lin L-C, Putnam FW (1978) Cold pepsin digestion: a novel method to produce the Fv fragment from human immunoglobulin M. Proc Natl Acad Sci USA 75:2649–2653

Lindner P, Guth B, Wülfing C, Krebber C, Steipe B, Müller F, Plückthun A (1992) Purification of native proteins from the cytoplasm and periplasm of *Escherichia coli* using IMAC and histidine tails: a comparison of proteins and protocols. Methods: a companion to Methods Enzymol 4:41–56

Liu J, Walsh CT (1990) Peptidyl-prolyl *cis-trans*-isomerase from *Escherichia coli*: a periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. Proc Natl Acad Sci USA 87:4028–4032

Lund J, Tanaka T, Takahashi N, Sarmay G, Arata Y, Jefferis R (1990) A protein structural change in aglycosylated IgG3 correlates with loss of huFcγRI and huFcγRIII binding and/or activation. Mol Immunol 27:1145–1153

Maeda H, Engel J, Schramm HJ (1976) Kinetics of dimerization of the variable fragment of the Bence-Jones protein Au. Eur J Biochem 69:133–139

Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J Mol Biol 222:581–597

Marquart M, Deisenhofer J, Huber R (1980) Crystallographic refinement and atomic models of the intact immunoglobulin molecule Kol and its antigen-binding fragment at 3.0 Å and 1.9 Å resolution. J Mol Biol 141:369–391

McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348:552–554

McCarthy JEG, Gualerzi C (1990) Translational control of prokaryotic gene expression. Trends Genet 6:78–85

McCartney JE, Tai M-S, Opperman H, Jin D, Warren FD, Weiner LM, Bookman MA, Stafford WF III, Houston LL, Huston JS (1993) Refolding of single-chain Fv with C-terminal cysteine (sFv′): formation of disulfide-bonded homodimers of anti-*c-erb*B-2 and anti-digoxin sFv′. ICSU Short Reports 13. ICSU, Stockholm

McManus S, Riechmann L (1991) Use of 2D NMR, protein engineering, and molecular modeling to study the hapten-binding site of an antibody Fv fragment against 2-phenyloxazolone. Biochemistry 30:5851–5857

Milstein C, Cuello AC (1983) Hybrid hybridomas and their use in immunohistochemistry. Nature 305:537–540

Morgan EL, Weigle WO (1987) Biological activities residing in the Fc region of immunoglobulin. Adv Immunol 40:61–134

Morrison SL (1992) In vitro antibodies: strategies for production and application. Annu Rev Immunol 10:239–265

Munro S, Pelham HRB (1986) An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 46:291–300

Neuberger MS, Williams GT, Fox RO (1984) Recombinant antibodies possessing novel effector functions. Nature 312:604–608

Nicholls PJ, Johnson VG, Andrew SM, Hoogenboom HR, Raus JCM, Youle RJ (1993) Characterization of single-chain antibody (sFv)-toxin fusion proteins produced in vitro in rabbit reticulocyte lysate. J Biol Chem 268:5302–5308

Nilson BHK, Solomon A, Björck L, Åkerström B (1992) Protein L from *Peptostreptococcus magnus* binds to the *K* light chain variable domain. J Biol Chem 267:2234–2239

Nisonoff A, Mandy WJ (1962) Quantitative estimation of the hybridization of rabbit antibodies. Nature 194:355–359

Orlandi R, Güssow DH, Jones PT, Winter G (1989) Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. Proc Natl Acad Sci USA 86:3833–3837

O'Shea EK, Rutkowski R, Kim PS (1989) Evidence that the leucine zipper is a coiled coil. Science 243:538–542

O'Shea EK, Klemm JD, Kim PS, Alber T (1991) X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. Science 254:543–544

Owen M, Gandecha A, Cockburn B, Whitelam G (1992) Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco. Biotechnology 10:790–794

Pabo CO, Suchanek EG (1986) Computer-aided model-building strategies for protein design. Biochemistry 25:5987–5991

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, 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

Pantoliano MW, Bird RE, Johnson S, Asel ED, Dodd SW, Wood JF, Hardman KD (1991) Conformational stability, folding, and ligand-binding affinity of single-chain Fv immunoglobulin fragments expressed in *Escherichia coli*. Biochemistry 30:10117–10125

Park JT (1987) The murein sacculus. In: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella typhimurium*. American Society for Microbiology, Washington, pp 23–30

Parmley SF, Smith GP (1988) Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene 73:305–318

Perez P, Hoffman RW, Shaw S, Bluestone JA, Segal DM (1985) Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody. Nature 316: 354–356

Perlmutter RM, Crews ST, Douglas R, Sorensen G, Johnson N, Nivera N, Gearhart PJ, Hood L (1984) The generation of diversity in phosphorylcholine-binding antibodies. Adv Immunol 35:1–37

Plückthun A (1993a) Antibody engineering to study protein-ligand interactions and catalysis: the phosphorylcholine binding antibodies. Front Bioorg Chem 3:26–65

Plückthun A (1993b) Stability of engineered antibody fragments in: *Stability and Stabilization of Enzymes* (van den Tweel WJJ, Harder A and Buitelaar RM, eds.) Elsevier Science Publishers, pp 81–90

Plückthun A, Skerra A (1989) Expression of functional antibody Fv and Fab fragments in *Escherichia coli*. Methods Enzymol 178:497–515

Podhajska AJ, Hasan N, Szybalski W (1985) Control of cloned gene expression by promoter inversion in vivo: construction of the heat-pulse-activated att-nutL-p-att-N module. Gene 40:163–168

Pollitt S, Zalkin H (1983) Role of primary structure and disulfide bond formation in β-lactamase secretion. J Bacteriol 153:27–32

Prickett KS, Amberg DC, Hopp TP (1989) A calcium-dependent antibody for identification and purification of recombinant proteins. Biotechniques 7:580–589

Pugsley AP (1993) The complete general secretory pathway in gram-negative bacteria. Microbiol Rev 57:50–108

Raso V, Griffin T (1981) Hybrid antibodies with dual specificity for the delivery of ricin to immunoglobulin-bearing target cells. Cancer Res 41:2073–2078

Regan L, DeGrado WF (1988) Characterization of a helical protein designed from first principles. Science 241:976–978

Reth M, Imanishi-Kari T, Rajewsky K (1979) Analysis of the repertoire of anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibodies in C 57 BL/6 mice by cell fusion. II. Characterization of idiotopes by monoclonal anti-idiotope antibodies. Eur J Immunol 9:1004–1013

Riechmann L, Foote J, Winter G (1988) Expression of an antibody Fv fragment in myeloma cells. J Mol Biol 203:825–828

Riesenberg D (1991) High-cell-density cultivation of *Escherichia coli*. Curr Opin Biotechnol 2:380–384

Rowe ES (1976) Dissociation and denaturation equilibria and kinetics of a homogeneous human immunoglobulin Fab fragment. Biochemistry 15:905–916

Rowe ES, Tanford C (1973) Equilibrium and kinetics of the denaturation of a homogeneous human immunoglobulin light chain. Biochemistry 12:4822–4827

Rudolph R (1990) Renaturation of recombinant, disulfide-bonded proteins from "inclusion bodies". In: Tschesche H (ed) Modern methods in protein- and nucleic acid research. de Gruyter, Berlin, pp 149–171

Russel M (1991) Filamentous phage assembly. Mol Microbiol 5:1607–1613

Sastry L, Alting-Mees M, Huse WD, Short JM, Sorge JA, Hay BN, Janda KD, Benkovic SJ, Lerner RA (1989) Cloning of the immunological repertoire in *Escherichia coli* for generation of monoclonal catalytic antibodies: construction of a heavy chain variable region-specific cDNA library. Proc Natl Acad Sci USA 86:5728–5732

Satow Y, Cohen GH, Padlan EA, Davies DR (1986) Phosphocholine binding immunoglobulin Fab McPC603. An X-ray diffraction study at 2.7 Å. J Mol Biol 190:593–604

Savage P, So A, Spooner RA, Epenetos AA (1993) A recombinant single-chain antibody interleukin-2 fusion protein. Br J Cancer 67:304–310

Sawyer JR, Tucker PW, Blattner FR (1992) Metal-binding chimeric antibodies expressed in *Escherichia coli*. Proc Natl Acad Sci USA 89:9754–9758

Schmidt TGM, Skerra A (1993) The random peptide library-assisted engineering of a C-terminal affinity peptide, useful for the detection and purification of a functional Ig Fv fragment. Protein Eng 6:109–122

Schoner BE, Belagaje RM, Schoner RG (1990) Enhanced translational efficiency with two-cistron expression system. Methods Enzymol 185:94–103

Schumaker VN, Poon PH (1990) Activation of the classical and alternative pathways of complement by immune complexes. In: Metzger H (ed) Fc receptors and the action of antibodies. American Society for Microbiology, Washington, pp 181–207

Scott JK, Smith GP (1990) Searching for peptide ligands with an epitope library. Science 249:386–390

Seetharam S, Chaudhary VK, FitzGerald DJ, Pastan I (1991) Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL. J Biol Chem 266:17376–17381

Segal DM (1990) Antibody-mediated killing by leukocytes. In: Metzger H (ed) Fc receptors and the action of antibodes. American Society for Microbiology, Washington, pp 291–301

Sen J, Beychok S (1986) Proteolytic dissection of a hapten binding site. Proteins 1:256–262

Sharon J, Givol D (1976) Preparation of the Fv fragment from the mouse myeloma XRPC-25 immunoglobulin possessing anti-dinitrophenyl activity. Biochemistry 15:1591–1594

Shibui T, Munakata K, Matsumoto R, Ohta K, Matsushima R, Morimoto Y, Negahari K (1993) High-level production and secretion of a mouse-human chimeric Fab fragment with specificity to human carcino embryonic antigen in *Escherichia coli*. Appl Microbiol Biotechnol 38:770–775

Shin S-U, Wright A, Bonagura V, Morrison SL (1992) Genetically-engineered antibodies: tools for the study of diverse properties of the antibody molecule. Immunol Rev 130:87–107

Skerra A, Plückthun A (1988) Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. Science 240:1038–1041

Skerra A, Plückthun A (1991) Secretion and in vivo folding of the Fab fragment of the antibody McPC603 in *Escherichia coli*: influence of disulphides and cis-prolines. Protein Eng 4:971–979

Skerra A, Pfitzinger I, Plückthun A (1991) The functional expression of antibody Fv fragments in *Escherichia coli*: improved vectors and a generally applicable purification technique. Biotechnology 9:273–278

Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228:1315–1317

Smith MC, Cook JA, Smanik PA, Wakulchik M, Kasher MS (1992) Kinetically inert Co(III) linkage through an engineered metal binding site: specific orientation of recombinant human papillomovirus type 16 E7 protein on a solid support. Methods: A companion to Methods Enzymol 4:73–78

Söderlind E, Simonsson Lagerkvist AC, Dueñas M, Malmborg AC, Ayala M, Danielsson L, Borrebaeck CAK (1993) Chaperonin assisted phage display of antibody fragments on filamentous bacteriophages. Biotechnology 11:503–507

Staerz UD, Kanagawa O, Bevan MJ (1985) Hybrid antibodies can target sites for attack by T cells. Nature 314:628–631

Stemmer WPC, Morris-SK, Kautzer CR, Wilson BS (1993a) Increased antibody expression from *Escherichia coli* through wobble-base library mutagenesis by enzymatic inverse PCR. Gene 123:1–7

Stemmer WPC, Morris SK, Wilson BS (1993b) Selection of an active single chain Fv antibody from a protein linker library prepared by enzymatic inverse PCR. Biotechniques 14:256–265

Stevens FJ, Westholm FA, Solomon A, Schiffer M (1980) Self-association of human immunoglobulin κI light chains: role of the third hypervariable region. Proc Natl Acad Sci USA 77:1144–1148

Stewart DE, Sarkar A, Wampler JE (1990) Occurrence and role of *cis* peptide bonds in protein structures. J Mol Biol 214:253–260

Sutton BJ, Phillips DC (1983) The three-dimensional structure of the carbohydrate within the Fc fragment of immunoglobulin G. Biochem Soc Trans 11:130–132

Tai M-S, Mudgett-Hunter M, Levinson D, Wu G-M, Haber E, Oppermann H, Huston JS (1990) A bifunctional fusion protein containing Fc-binding fragment B of staphylococcal protein A amino terminal to antidigoxin single-chain Fv. Biochemistry 29:8024–8030

Takagi H, Morinaga Y, Tsuchiya M, Ikemura H, Inouye M (1988) Control of folding of proteins secreted by a high expression secretion vector, pIN-III-*ompA*: 16-fold increase in production of active subtilisin E in *Escherichia coli*. Biotechnology 6:948–950

Takahashi H, Igarashi T, Shimada I, Arata Y (1991) Preparation of the Fv fragment from a short-chain mouse IgG2a anti-dansyl monoclonal antibody and use of selectively deuterated Fv analogues for two-dimensional ^{1}H NMR analyses of the antigen-antibody interactions. Biochemistry 30:2840–2847

Takkinen K, Laukkanen ML, Sizmann D, Alfthan K, Immonen T, Vanne L, Kaartinen M, Knowles JKC, Teeri TT (1991) An active single-chain antibody containing a cellulase linker domain is secreted by *Escherichia coli*. Protein Eng 4:837–841

Tao M-H, Morrison SL (1989) Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. J Immunol 143:2595–2601

Tomlinson IM, Walter G, Marks JD, Llewelyn MB, Winter G (1992) The repertoire of human germline V_H sequences reveals about 50 groups of V_H segments with different hypervariable loops. J Mol Biol 227:776–798

Trandinh CC, Pao GM, Saier MH (1992) Structural and evolutionary relationships among the immunophilins: two ubiquitous families of peptidyl-prolyl cis-trans isomerases. FASEB J 6:3410–3420

Wanner BL (1987) Phosphate regulation of gene expression in *Escherichia coli*. In: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmolella typhimurium*. American Society for Microbiology, Washington, pp 1326–1333

Ward ES, Güssow D, Griffiths AD, Jones PT, Winter G (1989) Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. Nature 341:544–546

Weir DM (ed) (1986) Immunochemistry. Blackwell, Oxford (Handbook of experimental immunology, vol 1, 4th edn)

Weissman JS, Kim PS (1991) Reexamination of the folding of BPTI: predominance of native intermediates. Science 253:1386–1393

Wels W, Harwerth I-M, Zwickl M, Hardman N, Groner B, Hynes NE (1992a) Construction, bacterial expression and characterization of a bifunctional single-chain antibody-phosphatase fusion protein targeted to the human erbB-2 receptor. Biotechnology 10:1128–1132

Wels W, Harwerth I-M, Mueller M, Groner B, Hynes NE (1992b) Selective inhibition of tumor cell growth by a recombinant single-chain antibody-toxin specific for the erbB-2 receptor. Cancer Res 52:6310–6317

Whitlow M, Filpula D (1991) Single-chain Fv proteins and their fusion proteins. Methods: a companion to Methods Enzymol 2:97–105

Whitlow M, Filpula D, Rollence ML, Feng SL, Wood JF (1994) Multivalent Fvs: characterization of single-chain Fv oligomers and preparation of a bispecific Fv. Protein Eng, in press

Wood CR, Boss MA, Patel TP, Emtage JS (1984) The influence of messenger RNA secondary structure on expression of an immunoglobulin heavy chain in *Escherichia coli*. Nucleic Acids Res 12:3937–3950

Wu XC, Ng SC, Near RI, Wong SL (1993) Efficient production of a functional single-chain antidigoxin antibody via an engineered *Bacillus subtilis* expression secretion system. Biotechnology 11:71–76

Wülfing C, Plückthun A (1993) A versatile and highly repressible E. coli expression system based on invertible promoters: expression of a gene encoding a toxic product. Gene 136:199–203

Wünsch E, Moroder L, Göhring-Romani S, Musiol H-J, Göhring W, Bovermann G (1988) Synthesis of the bis-cystinyl-fragment 225–232/225′–232′ of the human IgG1 hinge region. Int J Pept Protein Res 32:368–383

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

Yokota T, Milenic DE, Whitlow M, Schlom J (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res 52:3402–3408

Zeilstra-Ryalls J, Fayet O, Georgopoulos C (1991) The universally conserved GroE (Hsp60) Chaperonins. Annu Rev Microbiol 45:301–325