RECOMBINANT MICROBES FOR INDUSTRIAL AND AGRICULTURAL APPLICATIONS

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Escherichia coli Producing Recombinant Antibodies

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I. INTRODUCTION

Antibodies are a target of very diverse biotechnological interest. They are used widely in the laboratory, and hardly any branch of biology or biochemistry can do without them; be it for qualitative assays (e.g., Western blots, microdiffusion tests, or immunohistology), quantitative assays [radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs)], or immunoaffinity purification of compounds of interest. Even in organic chemistry, there has been interest in employing antibodies directed to suitably designed transition state analogs as catalysts [1–3]. In medicine, antibodies are of pivotal importance in diagnostics, generally in ELISA formats. In therapy, antibodies have been used as well, mostly still experimentally, and especially in the field of tumor therapy and diagnostics, there is currently great interest in antibody-based reagents [4]. For all these applications, different molecular properties of the antibodies are considered important, and different fragments may thus be appropriate. With the exception of whole antibodies, most of them can now be advantageously prepared in *Escherichia coli* in functional form [see Refs. 5, 6].

As all these applications have special needs, many antibody-based products had already been designed before the advent of gene technology. In most cases, manipulation of the structure was still carried out by proteolysis and chemical cross-linking. Here, the real advantage of recombinant DNA technology and bacterial expression sets in: not only can the antibody be produced reliably with comparatively little work in bacteria, but, with almost no extra effort after cloning, its gene can be modified.

In this review, first a brief consideration of the properties of various types of antibody fragments will be given. Then, the two main methods of expression will be discussed and compared: functional expression by secretion of the antibody fragment or preparation of inclusion bodies with subsequent in vitro refolding. New developments in screening for antigen-binding activities from libraries will be discussed. Finally, an overview of purification of recombinant antibody fragments produced in *E. coli* will be given.

II. ANTIBODY FRAGMENTS

Antibodies are multidomain proteins (Fig. 1) and, although only the variable domains are directly involved in binding, all domains have a purpose. If binding is all that is desired of the antibody fragment, the problem is reduced to producing the correctly assembled variable domains, V_H and V_L, in a stable heterodimeric association. A brief summary, which has appeared in more detailed form elsewhere [5,6], will be given about various antibody fragments.

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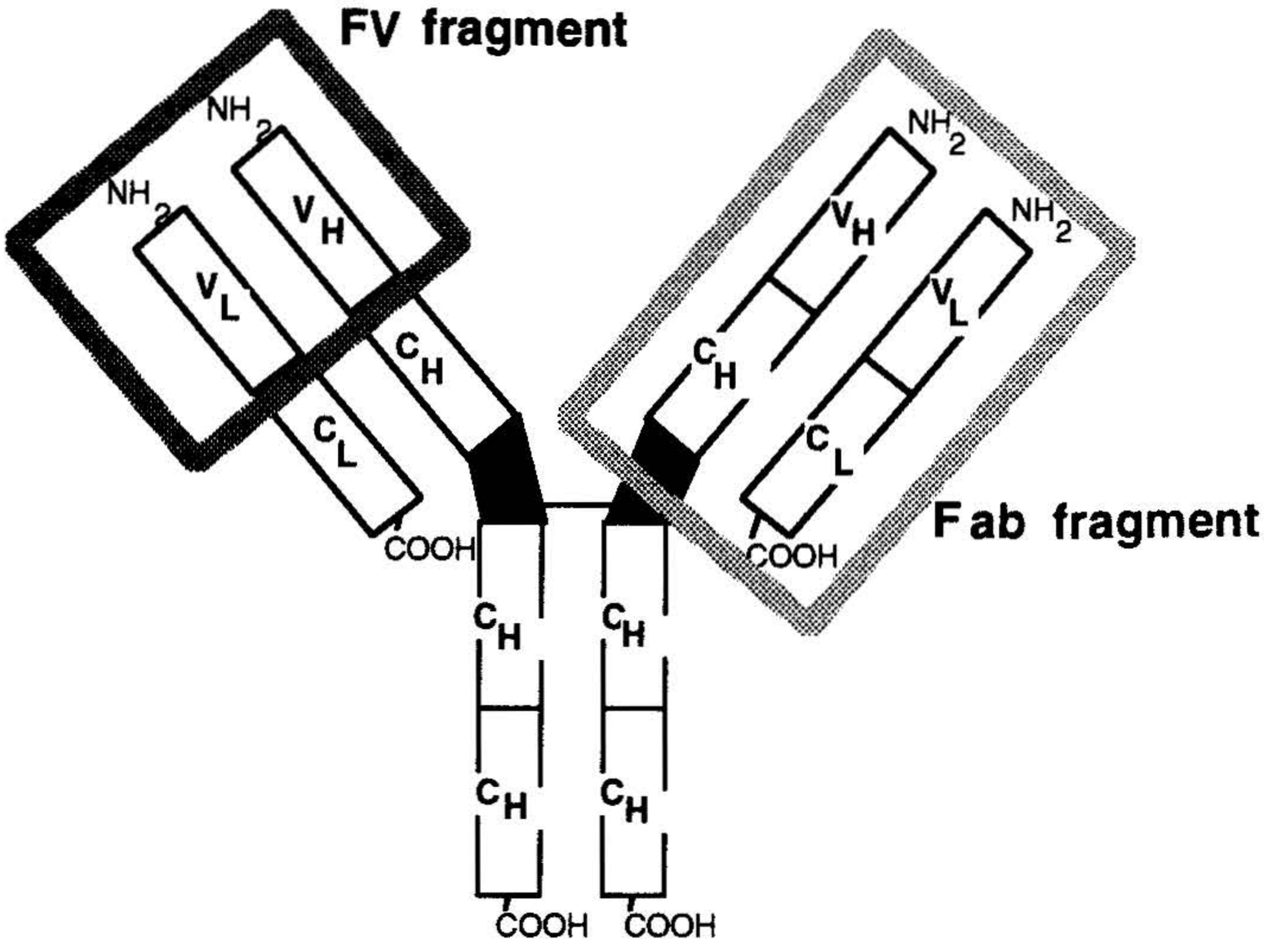


Figure 1 Schematic structure of an antibody of the IgG type. The Fab fragment and the Fv fragment are labeled. The variable domains have about 105–120 residues, the constant domains about 100 residues. All domains are β-barrels, with a centrally located intramolecular conserved disulfide bond, which is important for stability.

A. Whole Antibodies

The Fc fragment of many classes of whole antibodies (see Fig. 1) activates complement by binding to C1q and binds to Fc receptors, leading to responses such as antibody-dependent cellular cytotoxicity (ADCC) [7–10]. The binding to both C1q and to the Fc receptor depends on glycosylation of the Fc fragment [11–15]. As glycosylation is impossible in *E. coli*, these biological functions are not available for recombinant antibodies from *E. coli*; however, this glycosylation is of no importance for antigen binding [11–15]. Yet, there is currently no satisfactory bacterial expression system for (unglycosylated) whole native antibodies. One of the properties of whole antibodies, the bivalence (or even multivalence), can now be mimicked by "miniantibodies" (see later), which can be made in functional form in *E. coli* [16].

B. Fab Fragments

The Fab fragments are heterodimers comprising two variable domains and two constant domains [see Figs. 1 and 2]. Undoubtedly, the constant domains contribute to the association energy of the two chains for each other, and the association constant of a whole light chain (domains V_L and C_L) with the Fd fragment (domains V_H and C_H1) to give the Fab fragment is larger [17–19] than that of V_H and V_L to form an Fv fragment [20,21]. The Fab fragments can be made in functional form in *E. coli* [22,23].

C. Fv Fragments

The Fv fragments are the minimal fragments still containing the whole binding site (see Fig. 2). These fragments can be made in functional form in *E. coli* [24]. Depending on the particular

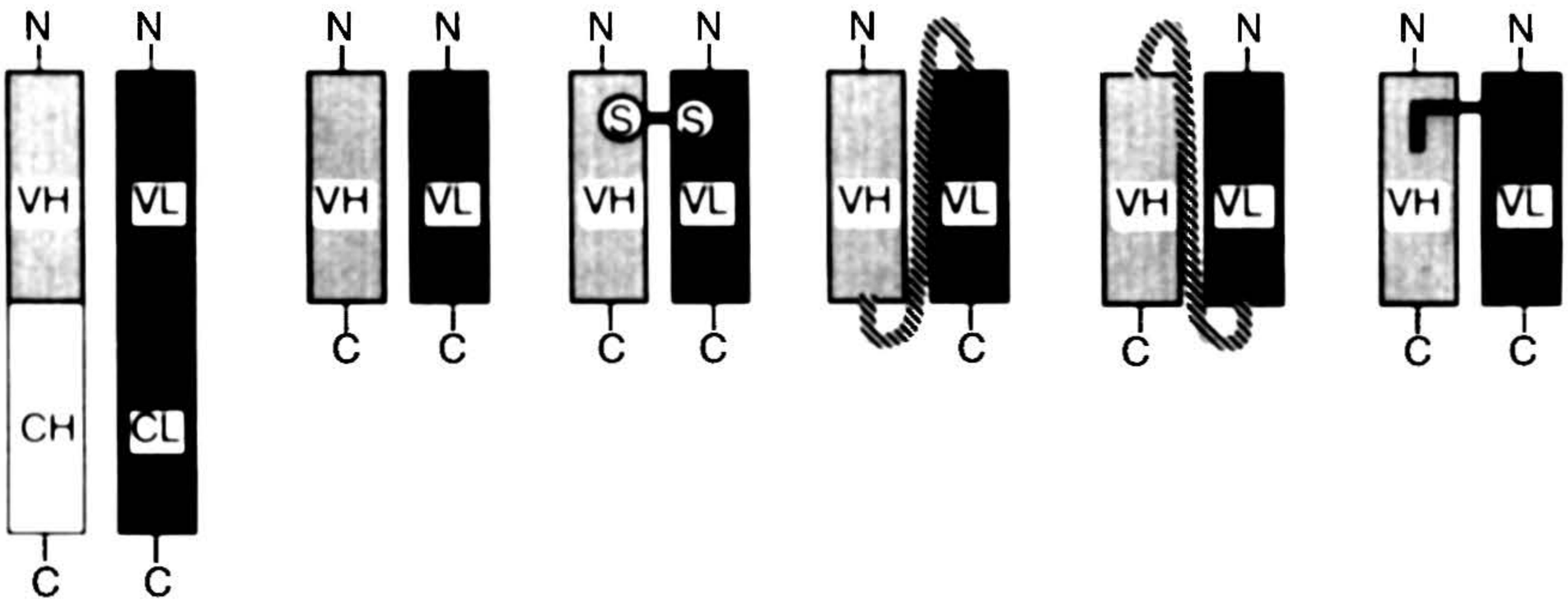


Figure 2 Monovalent fragments of antibodies functionally expressed in *E. coli*. (a) Fab fragment, (b) Fv fragment, (c) disulfide-linked Fv fragment, (d) single-chain Fv fragment with the orientation V_H–linker–V_L, (e) single-chain Fv fragment with the orientation V_L–linker–V_H, (f) Fv fragment that has been stabilized by chemical cross-linking after purification. (From Ref. 21)

antibody, they may be stable, or they can dissociate into V_H and V_L [21]. This dissociation can be counteracted by an engineered intermolecular V_H–V_L disulfide bond, by chemical cross-linking, or by connecting the two fragments genetically to form a so-called single-chain fragment [25, 26] (see next section).

D. Single-Chain Fv Fragments

Single-chain Fv fragments (see Fig. 2) are genetically linked either as V_H-linker-V_L or as V_L-linker-V_H [21,25–28]. Usually, linkers of about 15 amino acids have been used, and the linker $(Gly_4Ser)_3$ has been especially popular [27]. There seem to be no particular structural requirements for the linker, but its length must span the correct distance in space, and it must not disturb the folding of the two domains. Recently, it was shown by NMR that the $(Gly_4Ser)_3$ linker is very flexible and makes few contacts with the variable domains [32].

E. Single Domains And Single Loops

Although V_H domains have been advocated as substitutes of Fv fragments [29], their performance has been less than satisfactory because of poor solubility, lower affinity, and lower specificity [29,30]. They will not be considered further in this review. Synthetic peptides resembling antibody loops, although desirable in many applications, have not reached comparable binding efficiencies and, thus, are not yet useful for practical applications [31] and will not be considered here.

F. Bivalent Miniantibodies

Single-chain Fv fragments can be linked to form bivalent miniantibodies by fusing them at the COOH-terminus to a hinge region (to provide flexibility) and an amphipathic helix (to achieve dimerization) (Fig. 3). The helix has been taken either from a four-helix bundle design or from a leucine zipper [16 and references therein]. These bivalent miniantibodies assemble in the periplasm of *E. coli* and show the expected increase of avidity to surface-bound antigens [16].

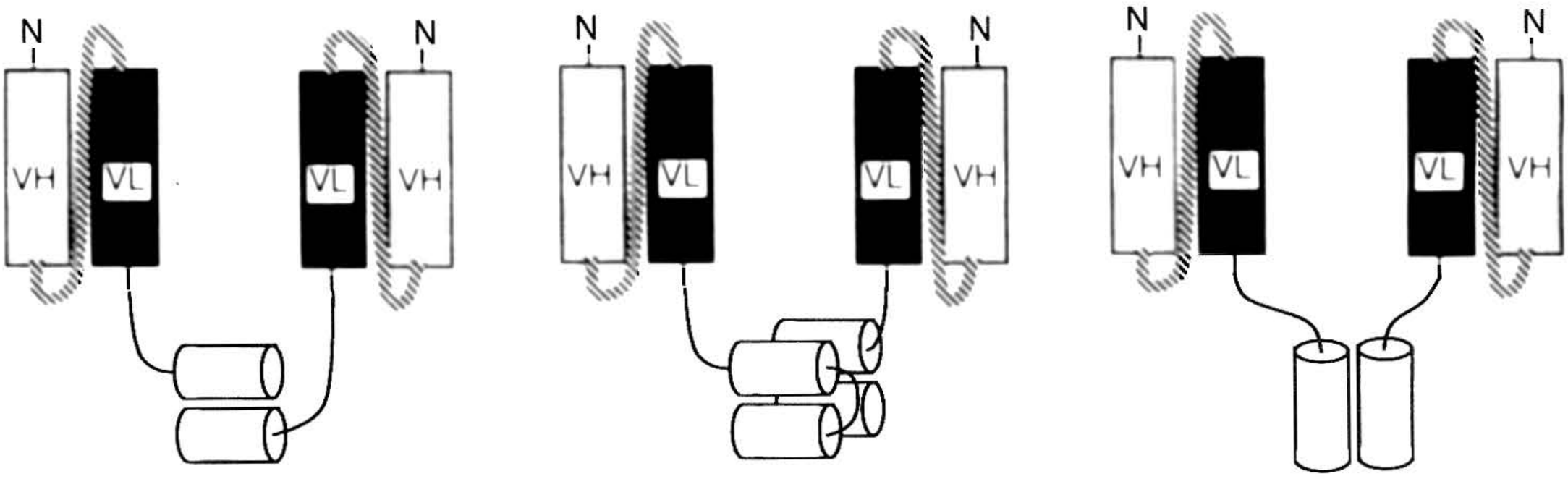


Figure 3 Bivalent fragments that have been shown to assemble in *E. coli* [16]. In each case, a scFv fragment is connected to a hinge region followed by an amphipathic helix. In (a) and (b) the helix comes from a four-helix bundle design by deGrado and co-workers. (a) Only one helix is fused, but the preponderant molecular species are dimers [16], not tetramers. (b) Two helices are fused in tandem and a four-helix bundle is probably obtained, as very stable dimers are formed in vivo. (c) A parallel coiled–coil helix from a leucine zipper is used. This last design is suitable for making heterodimers [for details, see Ref. 16].

III. WHY ENGINEER ANTIBODIES?

Why would one want to modify an antibody, if nature can apparently provide antibodies against any target and with high specificity and selectivity? A discussion of this question will lead to the definition of some desired specifications in the design of the appropriate antibody fragment for a particular application and this, in turn, will decide which expression strategy to pursue.

A. Decreasing the Size of a Fragment

The natural antibody has a given quaternary structure, depending on its subclass. Only a minor part of the antibody actually participates in binding (the variable domains) and the rest of the antibody has other functions, as previously discussed. In most applications, specificity of binding is the central goal, and any decrease in the size of the protein simply removes another potentially interfering interaction surface. Removal of the constant parts, however, requires alternative solutions to stability (see previous section; Fig. 2). It also requires designing other constant features for detection purposes and purification by other designed handles. Solutions to all these problems are now available [33–34]. Another feature that all antibody classes have in common is bivalence or even multivalence. However, there are now also solutions to this problem based on much smaller molecules [16], such that the size can be decreased without giving up bivalence (Fig. 3).

In clinical applications the small size of a single-chain Fv fragment of an antibody can also be of interest. Its distinctly different pharmacokinetics, having a much faster clearance rate, leads to different windows of selectivity and a better penetration of dense tumor tissue [35,36]. These factors can be important in tumor imaging and possibly tumor therapy. However, this field is still in its infancy, and only very few in vivo data are now available.

Fragments obtained by gene technology are of a defined size and require no further proteolytic processing and, consequently, genetic engineering is the preferred method of access to smaller fragments.

B. Decreasing the Immunogenicity

A decrease in immunogenicity is of interest for medical applications, because a repeated dose of a foreign antibody would lead to an immune response and, thereby, a reduction in efficiency of the foreign antibody and, in extreme cases, to severe health problems [37,38]. It is currently unknown whether the antigenicity of Fv fragments is already somewhat decreased because of their smaller size. Clearly, antibodies can be directed against the hypervariable regions (the anti-idiotypic response), but very little is known about the antigenic potency of any particular CDR sequence. Since most starting antibodies for clinical projects are still from mice, removing this antigenic potential is of great interest. To replace the constant domains by human ones (chimerization) would decrease the immunogenicity, but the murine variable domains are still immunogenic. The idea of "grafting" the complementarity-determining regions (CDR) of a mouse antibody onto a human framework [37] (humanization) would restrict the murine portion of the antibody to its absolute minimum, but numerous studies have now shown [summarized in Ref. 38] that, here, very often a loss of binding is observed if no further precautions are taken. The reasons lie in the many interactions between the framework and the CDRs, influencing the conformation of the CDRs. Although there are tabulations from crystallography [see, e. g., 39] that list contacts in each individual antibody, there is still some uncertainty about the need to change each particular framework residue, as the crystal structure of that particular antibody is usually unknown. Consequently, almost invariably several variants will have to be made and tested. Site-directed mutagenesis, with subsequent expression, is much faster in bacteria than it is in any other system.

C. Increasing Stability

The antibody domains are most likely optimized to pair in many different combinations and to permit the generation of a wide variety of specificities. Stability, on the other hand, requires the perfect match of the V_H–V_L surface of a particular pair and the optimal packing of the hydrophobic core of a particular domain with a particular set of CDRs. Thus, it is very probable that antibodies have not been optimized for stability. For instance, the free energy of folding of a V_L domain was only about 3.5 kcal/mol [A. Lupas, B. Steipe and A. Plückthun, unpublished]. An increase in the stability of antibody fragments is an area in which biotechnology can play an ever-increasing role. First steps in this direction have been taken by the introduction of additional disulfide bridges [21] (see Fig. 2).

D. Detection

Each of the applications discussed in the foregoing has its special needs for the design of the protein. In much of the diagnostics area, be it routine medical ELISAs or Western blots in the research laboratory, the antibody must somehow be detected. This can be done in three different ways:

1. An antibody or polyclonal antiserum against the recombinant one is raised which, in turn, is detected with antibodies coupled to an easily detectable enzyme, such as alkaline phosphatase, horse radish peroxidase, or luciferase [40]. The latter antiserum may also detect the recombinant one directly, if the recombinant one contains the same constant domains of the Fab portion, or the Fc part in a recombinant whole antibody to which the antiserum was raised. If, however, the recombinant antibody consists of only the variable domains, generally useful antisera suitable for detection will be rare. For the variable domains of nonrecombinant antibodies to be detected there is generally no alternative to raising a specific antiserum against the original antibody.

2. The second possibility of detecting the recombinant antibody requires its genetic modification. "Tag" or "flag" sequences can be attached to either end of the variable domain [29,33–34]. Two sequences have been useful: The sequence of the COOH-terminal decapeptide of the *myc* oncoprotein, to which a particular monoclonal antibody is available, can be attached to the COOH-terminus of the recombinant antibody fragment of interest and be detected by standard immunological techniques [29,41,42]. Alternatively, the sequence of a Ca^{2+}-binding motif of only four amino acids can be fused to the NH_2-terminus of the antibody and be detected with another specific antibody [43] (A. Knappik and A. Plückthun, unpublished). These two approaches obviate the need for constant domains as a means for immunodetection of the recombinant antibody, since much shorter peptides can fulfill the same purpose.

3. The most direct application of gene technology is in constructing fusion proteins containing an easily detectable enzyme. Here, the reporter enzyme is directly fused to the gene of the antibody of interest. This was first demonstrated in pioneering work using whole antibodies expressed in eukaryotes [44], but this technology can also be advantageously used with single-chain Fv fragments expressed in bacteria [45]. The most convenient fusion proteins are enzymes that can be easily expressed in bacteria and for which automated assays are available. Particularly, alkaline phosphatase falls into this category, since it is an *E. coli* enzyme; chromogenic [46] as well as very sensitive luminescence assays [47] are available; and this enzyme is active in fusion proteins [44,45].

E. Libraries

Recently, much effort has been spent on generating antibodies directly from libraries representing different types of variability. For instance, consensus primers have been designed to amplify antibody mRNA [see, e.g., 29,48,49]. Many other types of random mutations are currently being investigated to modify antibodies. In all these experiments, a screening step is necessary to find the antibody with the desired specificity in the library.

A particularly useful method is to randomly combine libraries of the heavy chain with those of the light chain, both obtained by the polymerase chain reaction (PCR) [50]. Each individual antibody molecule must still be made by secretion in functional form from *E. coli* in exactly the same way as described later for defined molecules. One possibility to generate the combinatorial library is to introduce an expression vector, constructed according to the principles described by Better et al. [22] and Skerra and Plückthun [24], into the genome of a λ phage [50]. The purpose of the phage is to provide a good transfection efficiency—in a sense a biological equivalent of electroporation. This is crucial for obtaining a full representation of clones of the combinatorial library. The phage-infected bacteria then produce the antibody while they are lysing, and the product can be detected in the plaques by probing with radioactive antigen, provided the antibody is of high affinity. Although useful for making the library and screening it, to actually produce an antibody fragment, killing of the bacteria by the phage is to be avoided. Consequently, the expression vector is excised from the phage again and periplasmic secretion is carried out with the plasmid as described in the following section.

The second consequence of functional secretion of antibodies from *E. coli* is the compatibility of the antibody folding with its display on the surface of a filamentous phage, such as M13 or fd. Since the NH_2-terminus of both the minor coat protein pIII (the product of gene *III*) and the major coat protein pVIII (product of gene *VIII*) are apparently exposed to the aqueous surrounding in the phage coat, extensions there are still compatible with phage assembly. During phage assembly, these coat proteins are present as intermediates in the inner membrane of *E. coli* [reviewed in Ref. 51], with their NH_2-termini exposed to the periplasmic space. Budding phage then coats its DNA with these proteins waiting in the membrane. Consequently, any protein-folding of fusion proteins,

consisting of NH_2-terminal antibody fragments and COOH-terminal phage coat proteins, would take place in the periplasm, but anchored to the inner membrane by the COOH-terminal membrane domain. Therefore, the same kind of fragments that correctly assemble in the periplasm can also ultimately be displayed on filamentous phage, with important applications in affinity screening of libraries. Such experiments have been carried out with both Fab fragments [52–57] and single-chain Fv fragments [58–60].

After finding a desired antibody in the library this antibody must then be produced in quantities, depending on the particular application, and perhaps a derivative, according to the requirements for detection, purification, stability, antigenicity, and such, must be engineered. For this purpose *E. coli* production is again most useful, as all screening steps are already carried out in *E. coli* or its phages.

IV. PRODUCTION OF ANTIBODY FRAGMENTS BY TRANSFORMED *ESCHERICHIA COLI*

There are currently two principal strategies for producing antibody fragments in *E. coli*: (1) by secreting the protein, leading directly to folded and assembled product (Fig. 4a) or (2) from inclusion bodies, with subsequent refolding in vitro (see Fig. 4b,c).

A. Expression by Secretion

Expression of antibody fragments by secretion in *E. coli* is an attempt to imitate the natural folding within the eukaryotic cell. There, both chains making up the antibody are transported to the endoplasmic reticulum, where disulfide formation and assembly of the quaternary structure occurs.

Conceptually, the requirement for correct assembly is straightforward: both chains making up the antibody need to be transported to the periplasm of the same cell. Presumably, each chain can act as the folding template for the other, and there is some evidence supporting this idea in that the presence of the light chain is required to prevent the precipitation of the heavy chain [61]. Additionally, in the oxidizing milieu, disulfide formation occurs. The cosecretion of both chains making up the antibody fragment can also be guaranteed by the covalent linking of both chains of the Fv fragment to form a single-chain Fv fragment [21].

Practically, the secretion of the antibody is stressful for *E. coli* and requires several precautions for successful production. If the cells have the opportunity to rid themselves of the plasmid (which may occur; e.g., by β-lactamase leaking out into the medium and destroying the antibiotic, most noticeably at 37°C), they will quickly be overgrown by plasmid-free cells [23]. Thus, other antibiotics are more useful and therefore, in our laboratory, kanamycin is now used in fermentations, and tetracycline has also been used successfully [62]. For the same reason, it is important to be able to turn off expression during the bacterial growth phase; hence tightly repressible promoters are advantageous. We have found the wild-type *lac* promoter useful [24,33], as it can be repressed not only by the omission of the inducer isopropyl-β-D-thiogalactoside (IPTG); but also by the addition of glucose [63].

The requirement for proper folding of the antibody demands cosecretion of both antibody chains, if they are not linked (Fig. 5). There are three possible solutions to this problem: (1) two plasmids, (2) two promoters on one plasmid, or (3) an artificial operon with one promoter. In principle, all three strategies should give functional antibody and all have now been used in phage-display vectors (see III. E), but practical reasons make the third one most attractive for production. As antibody secretion does present stress for *E. coli*, producing cells are at a growth disadvantage. Therefore, rare events, such as plasmid recombinations, may be selected for, and judging from our own observations with two-plasmid systems, this approach may

be less easily controllable, as unexpected plasmid losses or changes in copy number have occasionally be seen. Yet this is an area that has not been investigated systematically. Perhaps for somewhat similar reasons, the two-promoter strategy is not as easily carried out in practice. With the identical promoter, deletions may occur because of homologous recombinations within the plasmid. With two different promoters, however, it may be difficult to find conditions under which both are completely repressed. If both are strong (no matter whether they are identical or different) there may be more leakage from at least one of them. Nevertheless, a two-promoter system has been used successfully in a phage-display vector [57]. Again, there has been no systematic study comparing the three strategies.

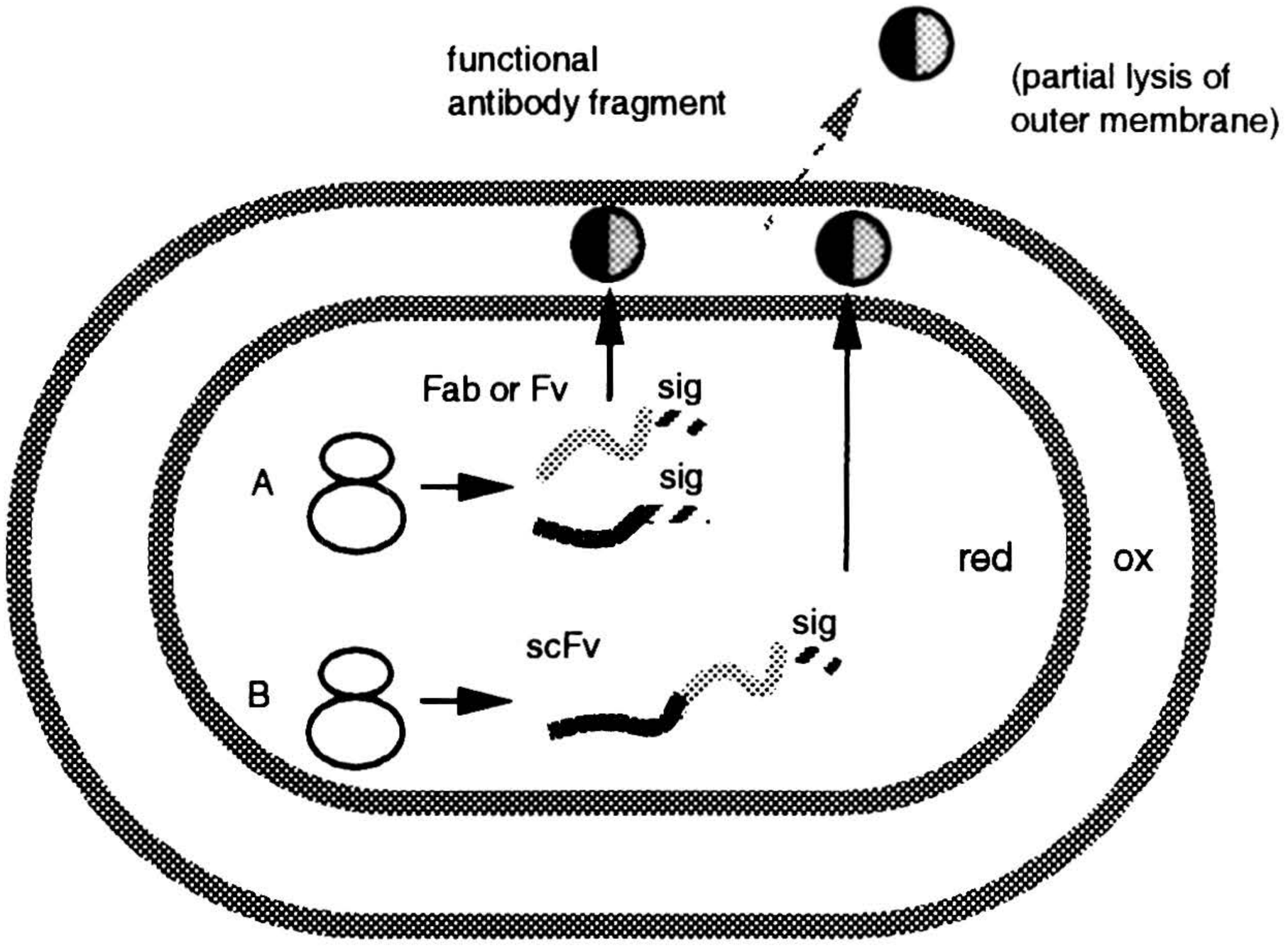
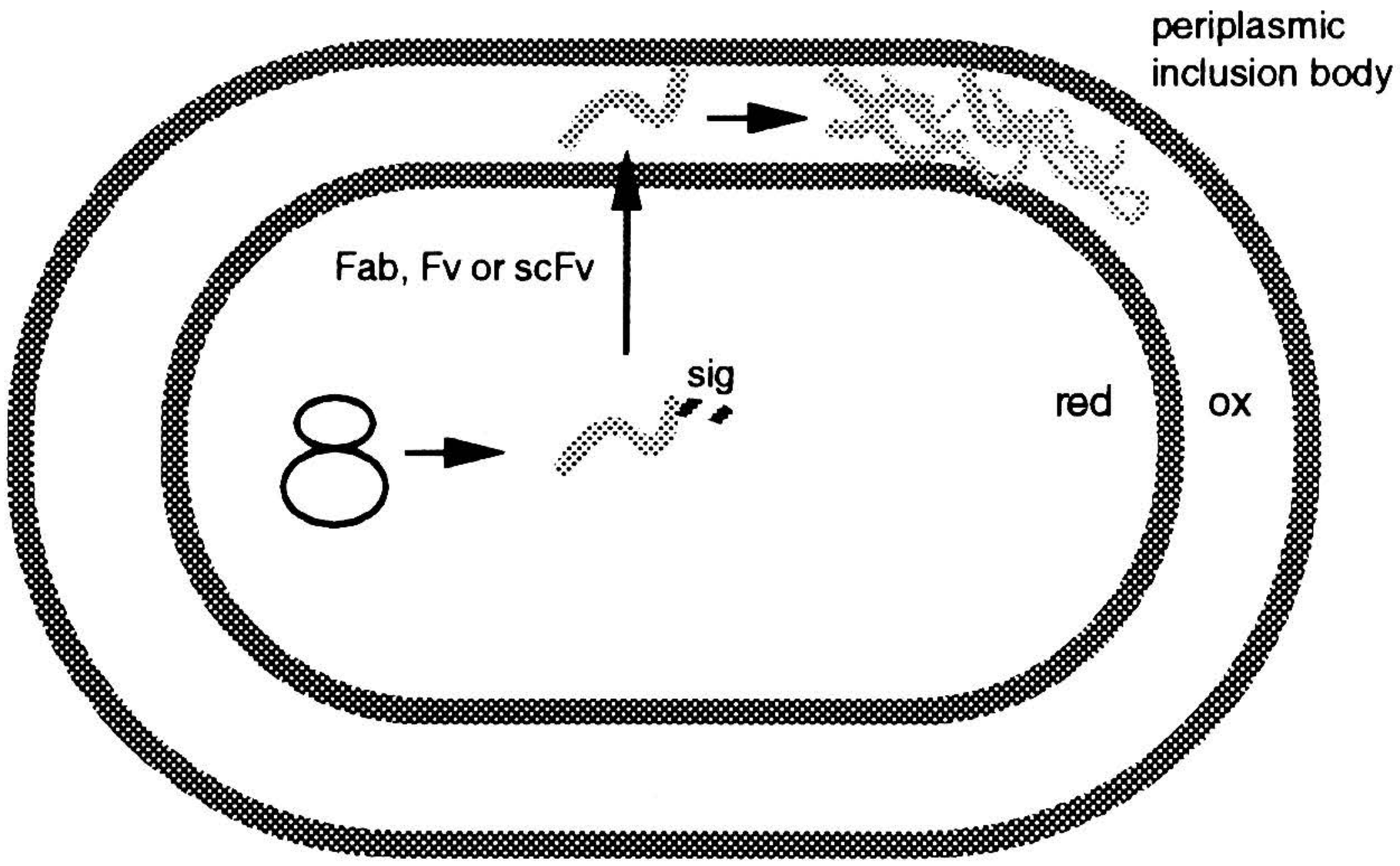
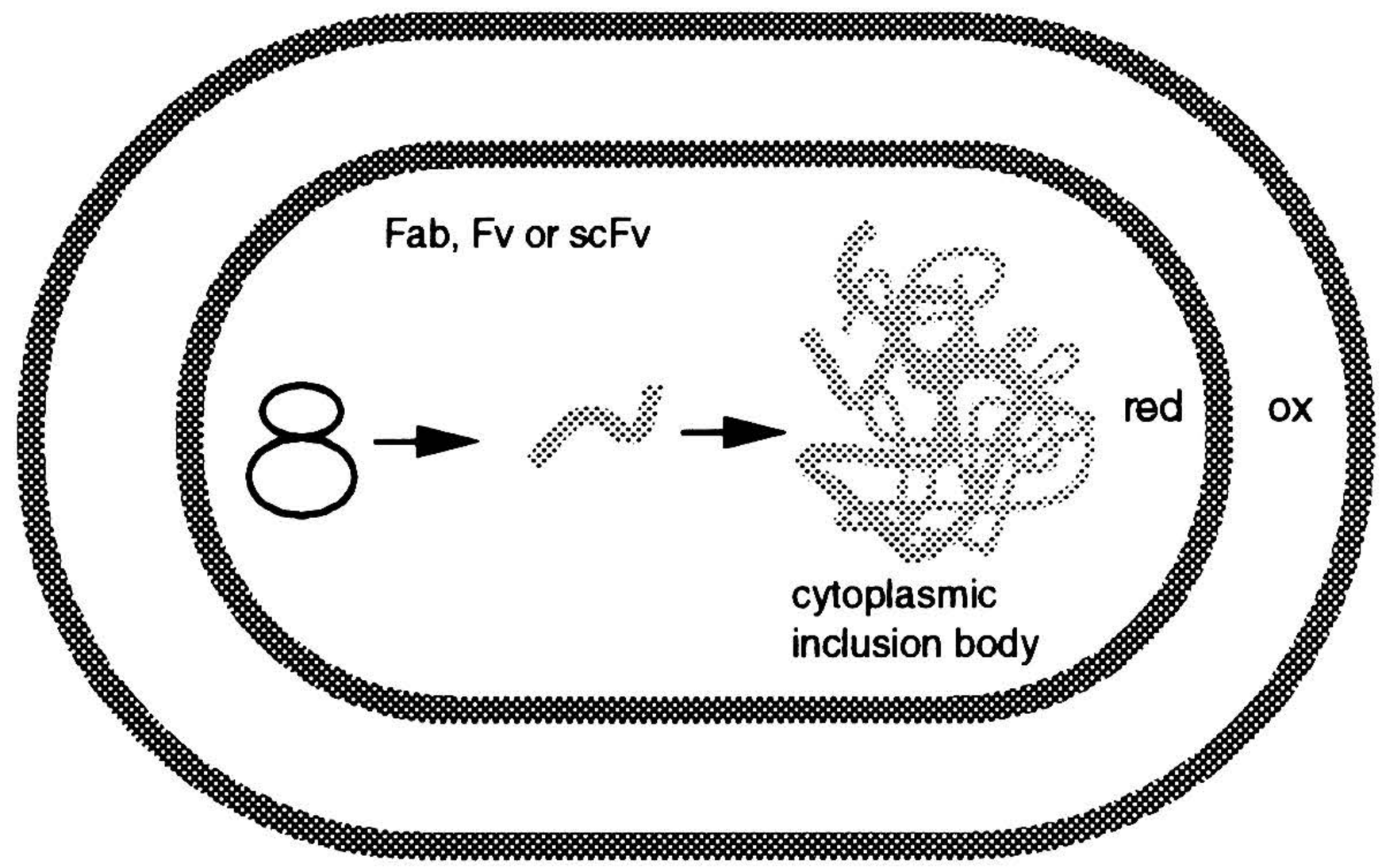


Figure 4 Schematic view of the three different expression strategies that have been useful for antibody production in *E. coli*. (a) Functional expression: here, the two chains making up the antibody combining site must be transported to the oxidizing milieu of the periplasm. In this compartment, there is a disulfide-forming enzyme (DsbA) that allows formation of the crucial intramolecular disulfide bonds that each domain has and requires 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. Alternatively, (B) both variable domains can be linked to form a continuous polypeptide chain (scFv fragment) that 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 lysis of the outer membrane after some time. This appears to occur independent of the type of antibody fragment and the signal sequence chosen although the primary sequence of the antibody can alter the extent. The phenomenon is observed more readily in shake flasks than in controlled fermentation conditions, during which 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 temperatures of 37°C or higher. The protein is apparently transported, processed, and then precipitates. The protein must be refolded in vitro. (c) Cytoplasmic inclusion body formation: Here, 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.





The most robust strategy to transport two chains to the periplasm is the cosecretion from a single cistron. This was first shown for an Fv fragment [24] and for an Fab fragment [22]. The vectors we use usually include a *lac* promoter, which is sufficiently strong and completely repressible. Alternatively, a *phoA* promoter has been used, as it can be cheaply induced by calculating the phosphate concentration in the growth medium to run out at the desired point [62]. The *phoA* system is efficiently repressed under conditions of high-phosphate concentrations in the medium, but it is more difficult to use on a laboratory scale. Since the phosphate content of rich media varies and is usually unknown, defined media are required.

The obvious intent in biotechnology is to optimize the yield. For this purpose, it is necessary to define the bottleneck in the process. There are several areas where a problem can conceivably lie: (1) plasmid copy number, (2) amount and stability of mRNA, (3) translation, (4) transport, (5) folding and assembly, (6) protein degradation, or combinations thereof.

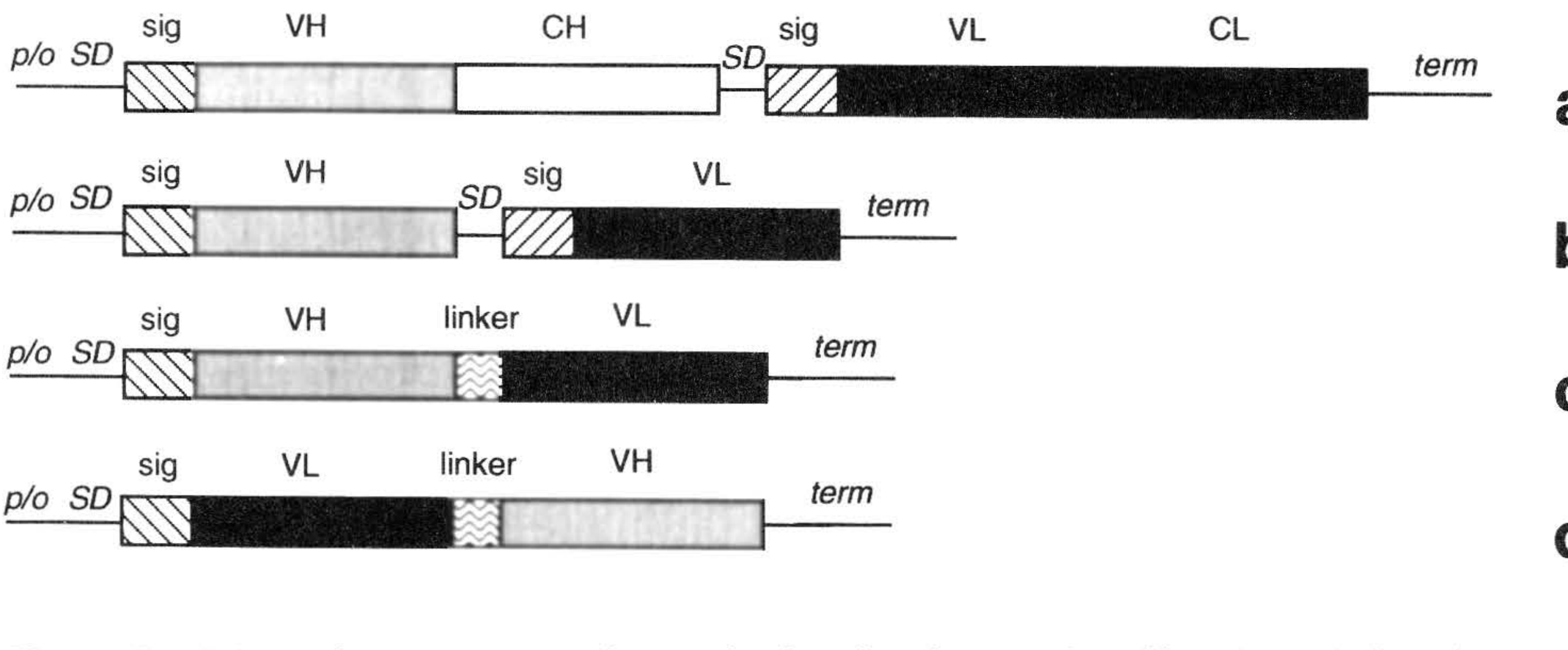


Figure 5 Schematic arrangement of genes for functional expression. Since both chains of the antibody must be secreted to the same periplasmic space to assemble, they must either be produced as two different secreted protein chains in the same cell (a,b) or they must be linked by a peptide linker (c,d). 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, *term* a transcription terminator, and the domains are labeled as in Figure 1.

1. Plasmid Copy Number

Many modern vectors, especially those based on modified ColE1 origins as in the pUC series [64], have high-copy numbers. The only problem not completely understood is the spontaneous decrease in plasmid copy numbers in *E. coli* cells that secrete proteins, imposing a stress on the cell. It remains to be investigated whether this is due to a decrease in the steady-state copy number or a total loss of plasmid in a subpopulation of cells. Indestructible antibiotics are helpful to combat this phenomenon, and independent plasmid-encoded functions (e.g., the plasmid-encoded antibiotic-resistance protein) can be used to measure this quantitatively.

2. Amount of Messenger RNA

The amount of mRNA is mostly determined by initiation frequency and degradation rate. The initiation rate (i. e., the promoter strength) is crucial, but easily remedied by the choice of any suitable "strong" promoter, because this is understood in much detail [65]. There is no indication that promoter strength is dependent on the gene itself. In principle, any strong promoter should be useful, but other criteria, such as repressibility, are much more restrictive. The mode of induction is also crucial. As will be discussed later, antibody-folding proceeds better at low temperature; accordingly, a heat-inducible promoter is less suitable for functional expression since heat pulses must then be used to carry out expression at room temperature. The degradation of mRNA is still poorly understood [66]. Therefore, even if an antibody gene was to be chemically synthesized completely, as has been done [67], it is unclear which features should be avoided or accumulated to increase the half-life of the message. Clearly, the sequence outside the coding region is important, too, although no particular rules can yet be given. There is currently no better strategy than to use 5' regions (i.e., the promoter and Shine–Dalgarno sequence) and 3' regions (i. e., the transcription terminator) of well-expressed genes to guard against exonucleolytic attack.

3. Translation

Translation efficiency is mostly determined by translation initiation [68]. Generally, elongation plays a smaller role, as the amounts of protein obtainable with secretory systems will reach other limits much sooner than that of the translation elongation. As a case in point, an antibody being functionally expressed at about 1 g/L (at high cell density of *E. coli*) [62] contained several rare codons, for which tRNAs are thought to be limiting.

Translation initiation is, despite intensive research, still not rationally understood. Yet, several trends have become apparent. Since translation initiation efficiency is a complex function of mRNA secondary structure, the Shine–Dalgarno sequence, its distance from the AUG start codon, the primary sequence of the 5'-untranslated region itself and even that of the beginning of the translated region [68], cassettes of other well-expressed genes are normally used, in which the whole sequence from the promoter to the start codon is left unchanged. For a transported gene, the signal sequence may also come from the same gene as the Shine–Dalgarno sequence. To increase translation efficiency further, the mini-cistron strategy can be used, in which a strongly expressed gene (e. g., β-galactosidase) is expressed only as a very short peptide, immediately followed by a stop codon and another Shine–Dalgarno sequence preceding the signal sequence of the codon of interest [24,33,69]. The intention is to gather ribosomes through the first and second ribosome-binding site, each one optimized in its natural context. However, other efficient upstream regions are now available that may be more potent than the two-cistron approach, although subsequent work using the extremely potent upstream region of gene *10* of phage T7 gave occasional problems of plasmid stability [Freund et al., unpublished results]. This region is extremely useful for cytoplasmic expression of inclusion bodies [32].

4. Transport

From the discussion to this point, it may seem desirable to increase both mRNA production and protein production as much as possible. Indeed, this seems to be harmful only if translation cannot be fully repressed during growth. Above a certain limit it is useless for functional expression, since protein-folding appears to limit the amount of soluble protein (see next section). This limit is not given by translocation through the membrane. When plasmids containing the w.t. *lac* promoter and the *lacUV5* promoter are compared, the amount of soluble protein remains remarkably constant. Yet, the amount of processed, but insoluble protein, dramatically increases [61]. In one instance [70], it has been directly demonstrated that this protein can be degraded by externally added proteinase after spheroplasting the cells. Therefore, most likely, this insoluble and processed protein has been transported and then precipitates. Only much smaller amounts of precursor can be seen, which cannot be degraded by externally added proteinase and, accordingly, must be cytoplasmic. This suggests that it is periplasmic folding, and not transport, that is the factor limiting the yield of folded protein. This hypothesis neglects the possibility that the amount of precursor detectable may not be representative, as it may be very efficiently degraded. On the other hand, it is hardly disputable that a major portion of the transported protein does not achieve the native state. Therefore, less protein can be folded than can be transported.

5. Folding and Assembly

From the previous discussion, it is most likely that protein-folding is the most decisive component determining the yield of final product. There are large variations in yields of different types of fragments when the same antibody is studied [62]. The Fab fragment gives a lower yield of folded material than the Fv or the single-chain Fv fragment, although the total amount of protein produced is much more similar, and the remaining portion is processed, but insoluble. These observations generated the idea that periplasmic folding steps might be responsible for the yield difference.

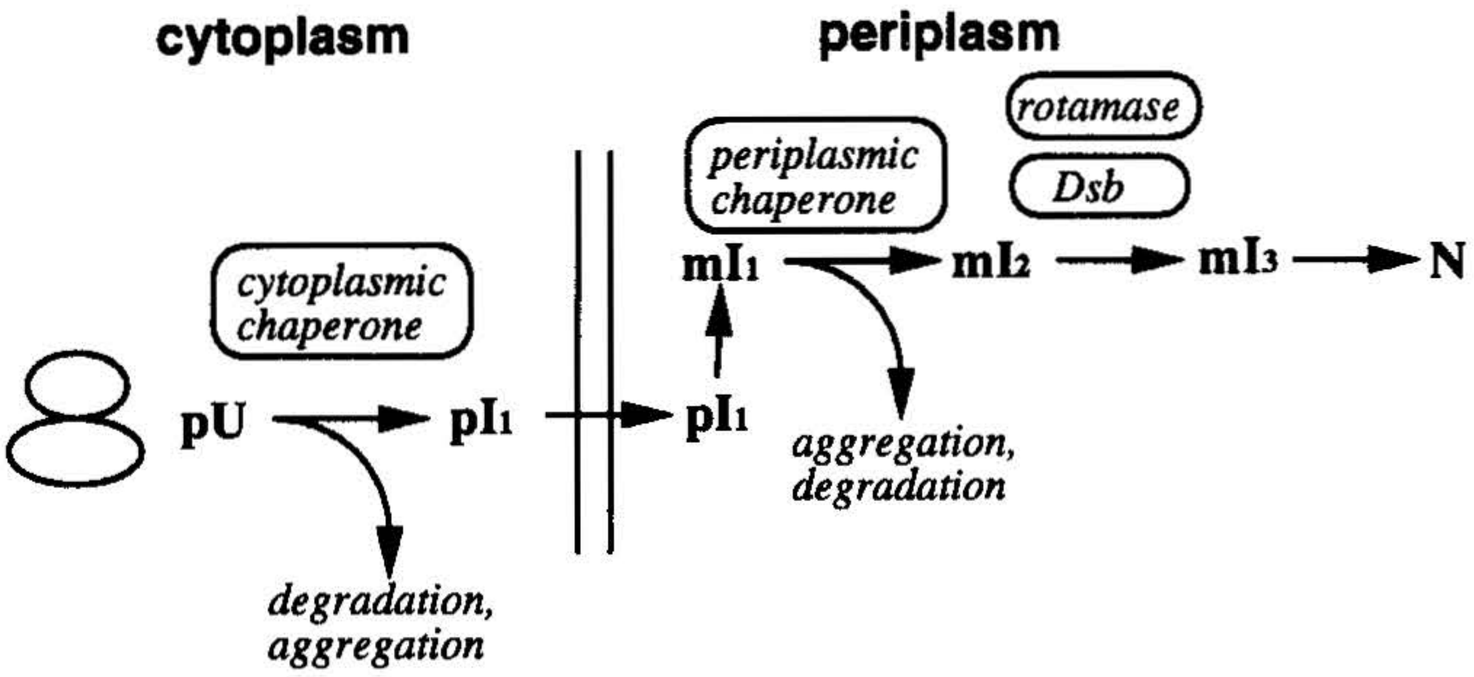


Figure 6 Hypothetical folding pathway of a secreted protein. The protein is made on the ribosome as an unfolded precursor (pU) and is probably kept in a transport-competent state (pI_1) by association with an as yet unidentified cytoplasmic factor (termed here *cytoplasmic chaperone*). It is unknown, 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 by other hypothetical intermediates (mI_2, mI_3) to the native state N. During periplasmic folding, a hypothetical yet unidentified periplasmic chaperone may act on the protein, and the disulfide-forming enzyme DsbA acts on the antibody [28]. 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, accordingly, the diversion to aggregates appears to happen before these steps, or to be independent of their extent.

A secreted protein probably emerges from the membrane in a largely unfolded state [71] (Fig. 6). The relevant protein-folding is, in this case, a periplasmic process. Consequently, it is not expected that the overexpression of cytoplasmic chaperones will be important. Their role for transported proteins seems to be to secure the unfolded state of the precursor to make it compatible for transport [72].

In contrast, slow periplasmic folding steps may increase the concentration of folding intermediates in the steady state and, thereby, lead to aggregation. Two types of slow-folding steps can be pinpointed: disulfide formation or rearrangement and proline *cis–trans* isomerization.

To test disulfide formation as a potentially limiting step, several disulfide bonds were first mutated away [61]. In the antibody McPC603, no functional Fab, Fv, or scFv fragment can be made if any of the disulfide bonds in the variable domains are missing [70]. In contrast, in an IgA, the C_H1 domain has an additional, dispensable S–S bond. If it is removed, however, the percentage of correctly assembled protein is not increased [61]. If the IgA is converted to an IgG1 by replacing the C_H1 domain, the expected interdomain S–S bond is produced, yet again, without dramatic change in yield [61].

In an additional series of experiments, the *E. coli* disulfide isomerase DsbA was then coexpressed [28]. In a *dsbA*$^-$ strain, almost no Fab fragment is made, clearly demonstrating that heterologous antibody protein makes use of DsbA and is even *dependent* on it. Plasmid encoded DsbA restores the function, which shows that it is functionally expressed. However, the overexpression does not increase the yield of folded material for the Fab, the Fv, or the scFv fragments [28]. Similar results are obtained by overexpressing the human disulfide isomerase with the Fab fragment (C. Krebber, A. Skerra, and A. Plückthum, unpublished).

Proline *cis–trans* isomerization was investigated as a potential folding limitation; decreasing the number of *cis*-prolines did not change the folding yield dramatically [61]. Neither did overexpression of the *E. coli* periplasmic proline *cis–trans* isomerase substantially change the amount of folded material for the Fab, Fv, or scFv fragments of the antibody McPC603 [28].

To test synergistic effects, DsbA and proline *cis–trans* isomerase were also both coexpressed

with the antibody fragment [28]. Furthermore, to "fill" the periplasm with these putative folding catalysts before antibody expression sets in, DsbA was also expressed under its own promoter, which appears to be constitutive. Finally, to facilitate disulfide isomerization, reduced glutathione was also added to the medium. Yet, there was never any sign of a significant change in folding yield [28].

In summary, it appears that there are aggregation steps taking place either before the formation of S–S bonds and isomerization of peptide bonds occurs, or the bonds are at least independent of the extent of the steps. Ongoing research is now directed at elucidating these steps.

6. Degradation

No comprehensive study on the effect of proteinases on various antibody fragments has yet been reported. It is likely that the recombinant fragments are being somewhat degraded by proteinases, but to various extents, and depending on the sequence. Occasionally, there are degradation products seen on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE); however, small peptide fragments may be removed much faster than they are made, and they probably do not accumulate [73]. Proteinase-deficient strains have been tested in our laboratory, yet without dramatic effects [O. Schroeckh and A. Plückthun, unpublished experiments].

Taking all these points into consideration, it is clear that further research is required to increase the productivity per cell. Yet, today high-density fermentation [74] can already be used to increase the productivity of cells. Preliminary experiments [P. Pack, M. Kujau, V. Schroeckh, D. Riesenberg and A. Plückthun, manuscript in preparation] look promising in that high-density fermentations are feasible for the secretory expression strategy. Future work will have to optimize these conditions.

In conclusion, this discussion shows that not all problems have yet been solved for the biotechnological production of antibodies by secretion. However, systematic research in this area is only now beginning, and already yields of 1 g/L bacteria are now obtainable in fermentation [62]. Very likely, there is ample room for improvement in the future, and this is an exceptionally simple procedure by which to obtain purified folded product.

B. Inclusion Bodies

The production of antibody proteins in *E. coli* as inclusion bodies differs little from the production of other recombinant proteins by this method. This was the first reported strategy for expressing antibody fragments in *E. coli* [75,76]. All types of monovalent antibody fragments (Fab, Fv, scFv, and even the chains for the whole antibody) have since been produced this way, and a variety of strains, plasmids, and promoters have been used. There are no apparent particular requirements for the strain or expression system specific for antibodies; any established production strain for *E. coli* inclusion bodies and most inducible strong promoters should be suitable. Fermentation of *E. coli* can also be carried out according to established principles [summarized in Ref. 74].

Too little is currently known to predict the ability of different sequences to form inclusion bodies (desired in this strategy) and in their susceptibility to proteinases. Recombinant proteins occasionally show signs of some degradation, 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. For example, it is not yet known whether proteinase-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, this 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 [77], but it has not been completely characterized for the extent of its disulfide formation and stability.

However, one may also isolate the portion of the protein that precipitates after transport [78] (Fig. 4c). At higher temperatures (37°C), the protein can still be transported, but the folding in the periplasm is severely impaired. Usually, to isolate soluble folded protein from the periplasm, growth must be carried out at low temperatures (see foregoing). Therefore, a heat-inducible promoter is incompatible with soluble expression, 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 proteinases. Therefore, some smaller 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 [78], but it is not known what percentage of molecules have them, and how many are correct.

Even though cell growth, vector construction, and inclusion body enrichment are straightforward (as there are no obvious specific differences from other recombinant proteins), refolding is not. However, if the protein is produced without a signal sequence, the 5'-coding region comes from the mature protein, not from the 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 [79]. In one example, even additional amino acids had to be fused to the heavy chain of the Fab fragment to obtain good inclusion body formation [80].

Several research groups have established protocols, and Fab fragments have been refolded at 10–40% yield [80], and scFv fragments have usually been refolded at 10–20% yield [25–27, 32,81,82]. After refolding, the protein must be purified again, and especially separated from incorrectly folded, but perhaps soluble, contaminating antibody protein [82]. 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 not uncommon to obtain a yield of only a low percentage of purified refolded protein [82] relative to the protein initially present in the inclusion body. In comparing the productivity of different strategies, it is crucial to keep this in mind.

What are the factors influencing the yield of refolding? Again, the refolding of antibodies is not, in principle, different from that of other disulfide-containing proteins [83]. First and foremost, the disulfide formation must be kinetically catalyzed and thermodynamically allowed. When using redox couples of reduced and oxidized glutathiones, concentrations of 1–2 mM reduced and 0.1–0.2 mM oxidized glutathione have been productive [80–83]. These conditions do allow the formation of the disulfide bonds thermodynamically, even if at first they appear to be "reducing," since the equilibrium of the protein disulfide bonds is intramolecular and dependent of the protein contribution to the folded state. Because of the importance of S–S formation, it is useful to carry out refolding at high pH 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. Thus, rather low protein concentrations have to be used, but the unfolded protein may be added stepwise, since the folded protein has a much higher solubility. Additionally, additives such as 1 M arginine are often useful, as they appear to increase the solubility of intermediates [83]. What is specific about arginine, however, remains mysterious. A protein concentration that is too low leads to gigantic volumes and losses in subsequent concentration steps [32, 80–83].

The addition of molecular chaperones in vitro has been attempted [81], yet without dramatic

effects, just as in vivo [28]. Slight improvements in yield are seen, but the effort of providing stoichiometric amounts of such proteins makes this approach forbidding on a technical scale.

All in all, in vitro refolding is a feasible strategy for a variety or antibody fragments. Yet, it is more laborious than production by secretion. Secretion is only now beginning to be optimized and often already at comparable levels [62], and if quantities of folded protein can be obtained by secretion similar to those in inclusion bodies, it will always be the method of choice. If one particular antibody fragment needs to be produced routinely, however, optimization of a refolding–purification scheme can be an attractive option.

V. PURIFICATION

The use of recombinant technology can also make purification of the fragments much easier. Conventionally, antibodies are purified by either (1) classic chromatography, not making use of any particular affinities of the antibody; (2) antigen affinity chromatography; or (3) affinity chromatography with bacterial antibody-binding proteins such as protein A, B, G or L [84–87].

Classic chromatography is usually not the prime choice, as it is laborious and not predictable for a new fragment.

Antigen affinity chromatography is very advantageous, if purified antigen is available, as it not only has a very high degree of selectivity, but also immediately separates folded from any unfolded material. For haptens, the elution can be carried out with soluble hapten under very mild conditions. For eluting protein antigens, the antibody–antigen interaction must be disturbed, and as the forces are the same that hold together the antigen itself and the antibody itself, a small amount of denaturation of the antibody can occur. This method is not applicable if antigen cannot be easily prepared in pure form, as frequently occurs (e.g., with tumor antigens or any other cell surface protein).

By using a phosphorylcholine affinity column, originally developed for the whole antibody, the recombinant Fab fragment, the Fv fragment, the scFv fragments (orientation V_H–linker–V_L and V_L–linker–V_H), and various bivalent miniantibodies, all could be purified directly from *E. coli* extracts [16,21,23,24,61] when the protein was functionally expressed by periplasmic secretion. Similarly, the same scFv fragment could be purified after in vitro refolding [32], as could the Fv fragment [70].

Because antibodies have the constant domains in common, several bacteria have developed proteins that bind to them. These, in turn, can be used when immobilized to a column to purify antibodies. However, proteins A, B, G, and L do not bind to all subclasses of all species [84–87], and most bind to domains in the Fc portion, although some Fab fragments and even variable domains are apparently recognized. Therefore, this method is in general limited to whole antibodies, which are not easily accessible from *E. coli*.

However, recombinant technology can now be used to create "purification handles." In principle, any easily purifiable protein domain can be used in a fusion protein. Because, usually, a large extraneous domain would be undesirable, a short peptide tail is the method of choice. Several peptide epitopes have been characterized to which monoclonal antibodies are available [see, e. g., 41–43]. Yet for a large-scale method, this is not economical.

The method of choice is the fusion of a short tail of histidines to the fragment of interest [33,34] (Fig. 7). Only a minimal perturbation is necessary, as five histidines are sufficient and, in a V_L domain, the last two accessible amino acids can be replaced by histidines, thereby requiring an addition of only three amino acids. This method has been tested for scFv fragments and V_L domains, and Fab fragments. Since the heavy chain of an Fab fragment is often insoluble, a

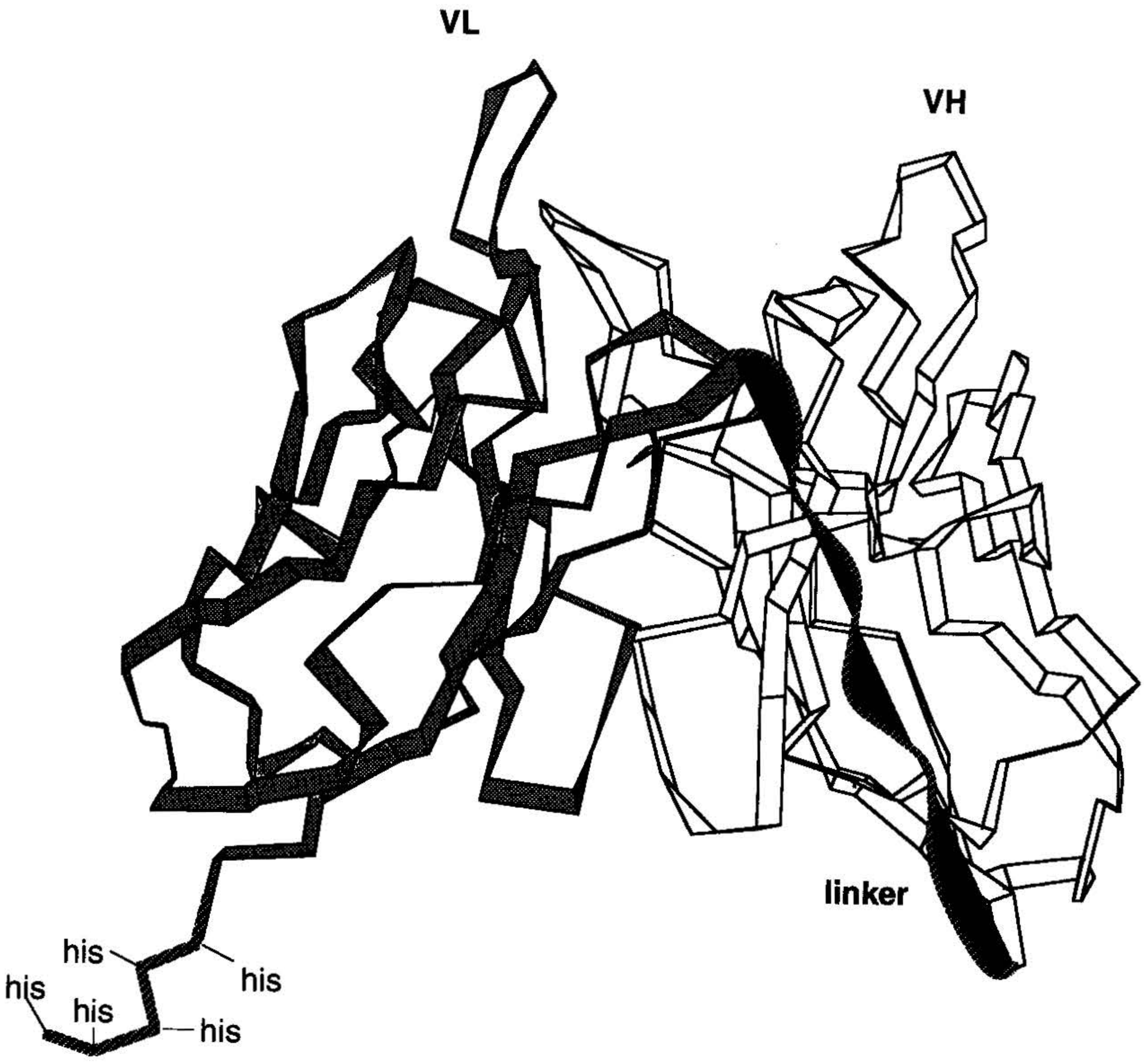


Figure 7 Schematic view of a single-chain Fv fragment containing a histidine tail for purification with immobilized metal-affinity chromatography [for details, see Refs. 33,34].

histidine tail there will selectively purify those chains that are correctly assembled with the light chain [A. Knappik, K. Bauer, and A. Plückthun, unpublished results]. Crystal structure analysis of the V_L domain of McPC603, with and without the tail, has shown that there is no structural change [34].

The purification is powerful enough to be carried out in one step from crude *E. coli* extracts. A variety of columns and buffers have been tested, and optimized procedures have been proposed [34]. There is circumstantial evidence that this method can even selectively purify correctly folded protein, as an incorrectly folded single-chain T-cell receptor, produced by the same method, does not bind to the column [34]. The most important criterion is undoubtedly the accessibility of the histidine tail. This method is also a safeguard against COOH-terminal degradation. Binding studies have shown the full functionality of the scFv fragment [33]. The histidine handle thus allows purification of antibody fragments from *E. coli* without the requirement for any constant domain or large affinity handles.

VI. CONCLUSIONS

The technology of screening, expressing, and modifying antibodies in *E. coli* will facilitate applications of antibodies in many areas of research, technology, and medicine. As this develop-

ment is still at its very beginning, it may reasonably be expected that biotechnology will be a most decisive factor in antibody-based reagents in the future.

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