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Antibody engineering: Comparison of bacterial, yeast, insect and mammalian expression systems

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Abstract

Engineered antibody molecules, and their fragments, are being increasingly exploited as scientific and clinical tools. However, one factor that can limit the applicability of this technology is the ability to express large amounts of active protein. In this review we describe the relative advantages and disadvantages of bacterial, yeast, insect and mammalian expression systems, and discuss some of the problems that can be encountered when using them. There is no 'universal' expression system, that can guarantee high yields of recombinant product, as every antibody-based molecule will pose its own problems in terms of expression. As a result the choice of system will depend on many factors, including the molecular species being expressed, the precise sequence of the individual antibody and the preferences of the individual investigator. However, there are general rules with regards to the design of expression vectors and systems which will help the investigator to make informed choices as to which strategy might be appropriate for their application.

Keywords: Antibody engineering; Expression systems; *Escherichia coli*; Yeast; Insect cells; Mammalian cells; Single-chain F

1. Introduction

The successful development of hybridoma technology by Köhler and Milstein (1975), and the resulting ability to produce monoclonal antibodies (MAbs) initiated a new era for science. Subsequently, the use of recombinant DNA technology, and the increasing knowledge of the genetics and structure of the immunoglobulins, has permitted the genetic manipulation of antibody molecules. This allows their properties to be altered, creating novel improved molecules. In order to do this, various expression systems have

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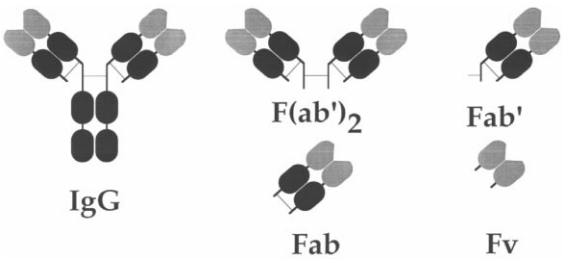


Fig. 1. Structure of the antibody molecule and its fragments. This figure shows the common antigen binding fragments of an IgG molecule. The IgG molecule is shown with the constant domains in dark shading and the variable domains of both the chains in lighter shading. The $F(ab')_2$ fragment can be made by pepsin digestion; following mild reduction this yields the Fab′ molecule. Fab fragments can be made by papain digestion. In some molecules it is possible to generate the Fv fragment by enzymatic approaches. Expression of the relevant gene segments also permits expression of recombinant versions of these molecules.

been developed with the aim of producing, at reasonable cost and effort, functional antigen binding molecules.

2. Recombinant antibodies

There are two main classes of recombinant antibodies. The first is based upon the intact immunoglobulin molecule (Fig. 1) and is designed to reduce the immunogenicity of the murine molecule. Thus chimeric molecules, which consist of the murine V regions and human constant regions, have been developed (Boulianne et al., 1984; Morrison et al., 1984; Neuberger et al., 1985; Better et al., 1988) as well as humanised antibodies in which just the CDRs are of rodent origin (Jones et al., 1986; Riechmann et al., 1988a).

Fig. 2. Recombinant molecules based on the Fv fragment. The Fv fragment is the smallest antibody fragment that retains an intact antigen binding site. However, it is unstable, as the V_H and V_L domains are free to dissociate. Two strategies have been adopted to overcome this. The first is to link the domains with a peptide to generate a single-chain Fv (sFv). The second is to introduce cysteines at the interface between the V_H and V_L domains, forming a disulphide bridge that holds them together [a disulphide stabilised Fv (dsFv)]. The location of the bond shown in the figure is for illustrative purposes only.

The second class of molecules consists of fragments of antibody molecules. These include fragments that are accessible through proteolysis, such as Fab, Fab′, $F(ab')_2$, as well as other fragments, such as Fv based molecules (Fig. 2). These molecules include sFv (single-chain Fv) (Bird et al., 1988; Huston et al., 1988), and the dsFv (disulphide stabilised Fv) (Glockshuber et al., 1990).

Small antibody fragments have advantages over whole immunoglobulins for some clinical applications, such as good penetration of solid tumours and rapid clearance (Huston et al., 1993, 1996). In addition they can be produced by phage display libraries (McCafferty et al., 1990).

These fragments can be endowed with new properties by fusion with other molecules, such as metal-binding proteins (George et al., 1995), cytokines (Boleti et al., 1995), toxins or drugs (Huston et al., 1993). They show particular promise for in vivo imaging applications, and radiolabelled sFv have been successfully used in the clinic to image colorectal carcinoma (Begent et al., 1996). In addition bispecific and bivalent antibodies can be made, such as diabodies (Holliger et al., 1993).

3. Expression of antibody molecules

Recombinant antibody fragments have been produced in various expression systems, such as bacterial (Better et al., 1988; Skerra and Plückthun, 1988; Huston et al., 1988; Bird et al., 1988), mammalian (Jost et al., 1994; Dorai et al., 1994) insect (Bei et al., 1995), yeast (Davis et al., 1991; Ridder et al., 1995b), plant (Whitelam et al., 1994) and *in vitro* translation systems (Nicholls et al., 1993).

In order to achieve a desirable expression, the cloned gene must be transcribed and translated efficiently. The yields and biological activity of recombinant proteins differ greatly, and depend on a large number of factors, such as solubility, stability and size of the protein.

Every protein poses unique problems in its expression because of its unique amino acid sequence. Although general conclusions can be

drawn from the study of one protein, expression has to be optimised for every new protein. While the term 'antibody' covers one class of protein, each antibody has a different sequence. Therefore the expression of each antibody or its fragments has its own problems. One expression system which may be suitable for the expression of one antibody may not be suitable for another. The optimal system will depend on the type of molecule being expressed (IgG, Fab, sFv, diabody), the individual antibody and also other factors, such as the required quantity and purity of the final product.

4. Gene expression using *Escherichia coli* cells

Immunoglobulin fragments are commonly expressed in *E. coli*. One advantage of this system is the ability to produce protein in large quantities. *E. coli* grow at a very fast rate in comparison to mammalian cells, giving the opportunity to purify, analyse and use the expressed protein in a much shorter time. In addition, transformation of *E. coli* cells with the foreign DNA is easy and requires minimal amounts of DNA. Antibody engineering using *E. coli* tends to be inexpensive. These reasons explain the popularity of bacterial systems. However, *E. coli* are not capable of glycosylating proteins. Therefore if whole antibody molecules are required, which are glycosylated in the C_H2 domain, it is necessary to use other expression systems.

In order to achieve successful expression, the gene encoding the antibody molecule must be placed in the context of appropriate sequences that allow transcription and translation of the protein. Inducible promoters are normally used to control expression of the protein. This is vital to prevent loss or mutation of the gene in situations where its production might be toxic to the bacteria. Commonly used promoters include the *lac* promoter, the *trp* promoter and their hybrid, the *tac* promoter that is regulated by the *lac* repressor and is induced by isopropyl-β-galactosidase (IPTG) (Amann et al., 1983; de Boer et al., 1983). Another popular promoter is the λP_L promoter, responsible for the transcription of the λ DNA molecule, which is regulated by a temperature-sensitive repressor. The T7 RNA promoter can also be used to obtain tightly controlled, high level, expression which involves two levels of amplification (Tabor and Richardson, 1985; Studier and Moffatt, 1986). A second important factor for efficient translation in *E. coli* is the existence of a prokaryotic ribosome-binding site (Gold et al., 1981). It consists of an initiation codon (ATG) and the Shine–Dalgarno sequence (SD), formed by 3–9 nucleotides and located 3–11 base pairs (bp) upstream from the initiation codon (Shine and Dalgarno, 1975; Steitz, 1979). The last important control element is the transcription terminator which prevents transcription beyond the desired gene and adds stability to the DNA.

The expression of recombinant antibody fragments in the reducing environment of the cytoplasm leads to the formation of insoluble inclusion bodies, which contain unfolded protein. This necessitates the development of refolding protocols to recover active material. There are a number of refolding strategies that can be employed (Fig. 3), and they need to be optimised for each molecule. Most strategies include the isolation of inclusion bodies, the solubilisation of the recombinant proteins, and their renaturation in an environment that promotes the correct disulphide bond formation and adoption of the appropriate three-dimensional shape. This review will not attempt to provide a detailed discussion of different refolding protocols, but rather will concentrate on the main principles of the process. Further detail can be found in Huston et al. (1995).

Solubilisation of the inactive proteins is typically done using denaturing agents, such as guanidine–HCl or urea. However, mild detergents, which do not bind too strongly to the protein (Tanford, 1968), can also be used (Lacks and Springhorn, 1980; Kurucz et al., 1995). In addition reducing agents, such as β-mercaptoethanol or dithiothreitol (DTT) can be used to reduce inter- and intra-chain disulphide bonds that might have formed during the lysis of the bacteria and solubilisation of the protein.

The formation of disulphide bonds can be performed by simple air oxidation (Anfinsen et al., 1961), in some cases promoted by the presence of metal ions (Saxena and Wetlaufer, 1970). 'Shuf-

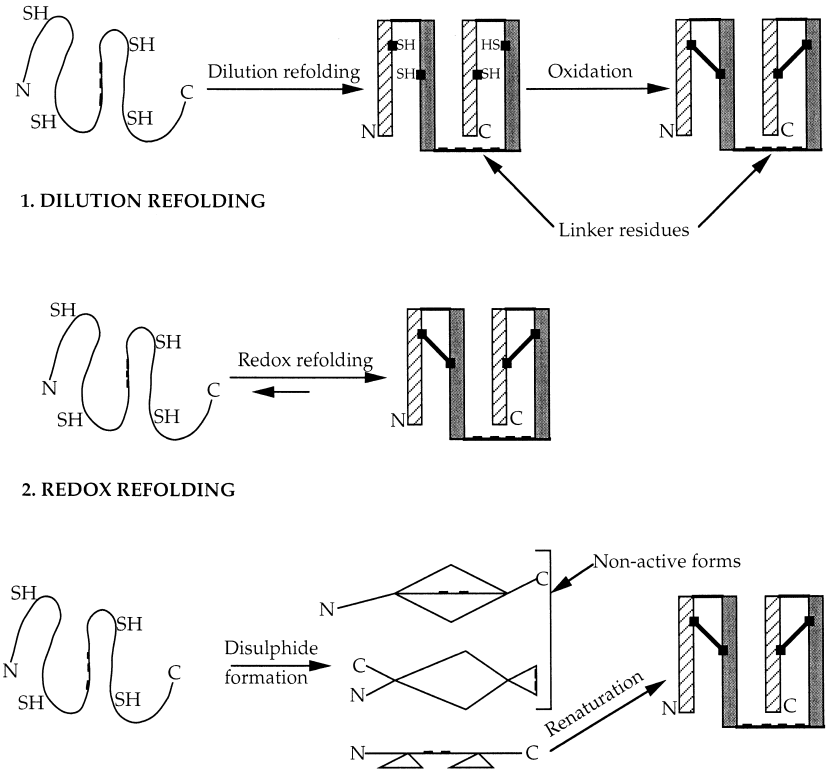
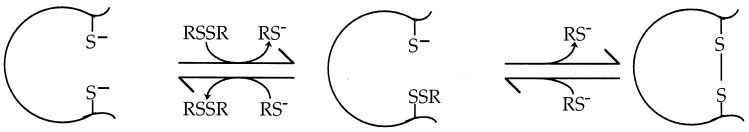


Fig. 3. Refolding pathways for sFv molecules. This figure illustrates the three major pathways by which denatured and reduced sFv molecules (as might be found after solubilisation and reduction from inclusion bodies) can be refolded. In all the figures, the fully denatured and reduced molecule is shown at the left, with the cysteine residues reduced. The linker residue is indicated by a crenulated line. The fully refolded molecules are shown to the right of the diagram. In dilution refolding (1) the denatured molecule is allowed to renature, usually by rapid dilution into a buffer that lacks the chaotropic agent responsible for denaturation. As the molecule has adopted the correct three-dimensional shape, oxidation of the cysteine residues leads to formation of the appropriate disulphide bonds. In redox refolding (2) the renaturation and reoxidation occur at the same time, forming an equilibrium with the denatured form of the molecule and other partially folded or incorrectly folded species. This approach relies on the correctly refolded molecule being the energetically preferred species, so becoming the predominant form. In disulphide restricted refolding (3) the disulphide bonds are allowed to form in a random manner. The molecule is then renatured. As two of the intermediate species will have incorrect disulphide bonds, they are unable to form an active sFv. An alternative form of disulphide restricted refolding can be performed on insoluble material obtained from the periplasm, where the disulphide bonds are assumed to be correctly formed, but the molecule has precipitated out of solution. Which of these schemes work is dependent on the properties of the individual sFv. For example, in some cases the correctly refolded molecule is not the most energetically stable, and so scheme 3 must be adopted. Figure adapted from Huston et al. (1995).

fling' (breaking and reforming) of the disulphide bonds to increase the chance of obtaining the correct configuration can be promoted by use of enzymes such as disulphide isomerase (Carmichael et al., 1977) or the inclusion of a redox couple made by a mixture of reduced and oxidised thiol groups (Saxena and Wetlaufer, 1970), as provided, for example, by glutathione (Fig. 4).

One of the major problems that needs to be overcome during the refolding process is the formation of aggregates. These are a consequence of the interaction of hydrophobic patches on the surface of malfolded or partially folded proteins. This process can be minimised by lowering the concentration of the refolding mixture.

Refolding can also be promoted by addition of



Number of disulphide bonds	*Number of combinations*
1	1
2	3
3	15
4	105
5	945
6	10395
7	135135
8	2027025
9	34459425
10	654729075
11	13749310575
12	316234143225
13	7905853580625
14	213458046676875
15	6190283353629375

Fig. 4. Formation of disulphide bonds during refolding. One of the major problems with refolding is to ensure that the cysteine residues in the molecule oxidise to form the correct disulphide bonds. At the bottom is shown the number of combinations of different disulphide bonds that can be formed in a molecule, depending on the number of bonds in the native molecule. As sFv fragments normally have two disulphide bonds, there are three different ways in which these bonds can be formed. If the bonds are formed at random only a third of the molecules will have their cysteines joined in the correct configuration. As can be seen the number of combinations rapidly rises with the number of disulphide bonds. One way to encourage the formation of the correct bonds is to set up a redox couple, for example by using a mixture of oxidised and reduced glutathione. These form equilibrium reactions, as shown at the top of the figure, which leads to the formation and breaking of disulphide bonds. This 'shuffling' can allow the incorrect combinations of disulphide bonds to be changed, and, assuming that the correct conformation is energetically favourable, lead to an increase in the correct combination. Figure adapted from Jaenicke and Rudolph (1989).

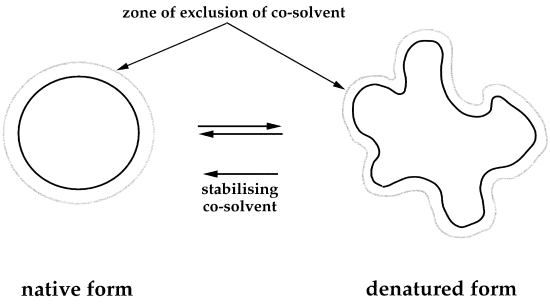


Fig. 5. Co-solvents. The addition of a stabilising co-solvent encourages the formation of the native structure of a protein during refolding (shifts the equilibrium to the left), as the zone of exclusion of the co-solvent is increased for asymmetric, denatured molecules. Figure adapted from Timasheff and Arakawa (1997).

a stabilising co-solvent, such as arginine. These co-solvents are excluded from the area immediately surrounding the protein, either due to steric hindrance (as is seen with polyethylene glycol) or by perturbation of the surface tension of water at the interface between the protein and the solvent (the mechanism by which arginine acts as a co-solvent), or by chemical interactions between the co-solvent and the protein (such as charge repulsion) (Timasheff and Arakawa, 1997). In mal-folded or unfolded proteins (which tend to be asymmetric) the area (or zone) in which the co-solvents are excluded is greater than the native molecule (Fig. 5). As the creation of zones of exclusion are thermodynamically unfavourable this encourages the correct folding of the molecules (Timasheff and Arakawa, 1997).

A recurring theme in this review is that every antibody or antibody fragment is unique. There is no one universal refolding protocol that can be

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