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Biotechnological Aspects of Antibody Production in E. coli

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Summary

The production of genetically engineered antibodies in *Escherichia coli* is now possible. The resulting fragments are completely functional and have antigen binding constants indistinguishable from the natural antibody. This article summarizes the biochemical basis of this newly developed technology and the properties of the resulting fragments. It is likely that this technology will have an important role in antibody production for technical, medical and research uses. Screening of *E. coli* libraries may mount a challenge to traditional antibody production methods.

Introduction

Antibodies are invaluable reagents in biotechnology. Today, ever since the discovery of monoclonal antibodies [1], the production of antibodies and of antibody derived reagents is itself a focus of biotechnology. The protocols for making hybridomas have continuously been streamlined, and the large scale production of monoclonal antibodies by the fermentation of hybridoma cells has evolved to processes with quite satisfactory yields [2]. The advent of gene technology has however added a new dimension to this field. It is now possible to generate recombinant antibodies not present in the natural repertoire. The key technology is the biotechnology of producing recombinant antibody molecules, and this review will emphasize the recently developed [3, 4] use of *E. coli* for this purpose, and the new possibilities resulting from this technology.

Initially, the production of genetically engineered antibodies was carried out in non-producing myeloma cells [5, 6], but non-lymphoid cells have been used as production hosts as well [7]. Recently, other eukaryotic hosts have also been described, such as baculovirus infected insect cells [8, 9], plants [10], and yeast [11]. The large scale techniques of fermentation of baculovirus infected cells are only now beginning to be developed [12], and not nearly as much empirical knowledge has yet been accumulated as with antibody production in mammalian cells. Yeast [11] has so far given only rather modest yields. It also remains to be seen, whether plants or plant cells will be a competitive production host, as they are not known for particularly convenient handling. While all these techniques will certainly be much improved, they must compete with the highly developed antibody fermentation using mammalian cells [5–7]. In cells derived from an antibody producing

animal, the glycosylation pattern is identical as in the animal, whereas this may not necessarily be the case in yeast, plants or insect cells. While glycosylation is not involved in antigen binding, it does influence several effector functions of the antibody (reviewed in [13]), and for medical applications, this may be an important consideration. Clearly, mammalian cells still have an important place in recombinant antibody production today, especially for therapeutic use.

In all applications, where antigen binding is the main focus, expression in *E. coli* is now a very viable alternative. Several years ago, we and others [3, 4, 14, 15], have developed the expression technology for correctly folded antibody fragments in *E. coli*, which now have opened the door to completely new types of biotechnological approaches.

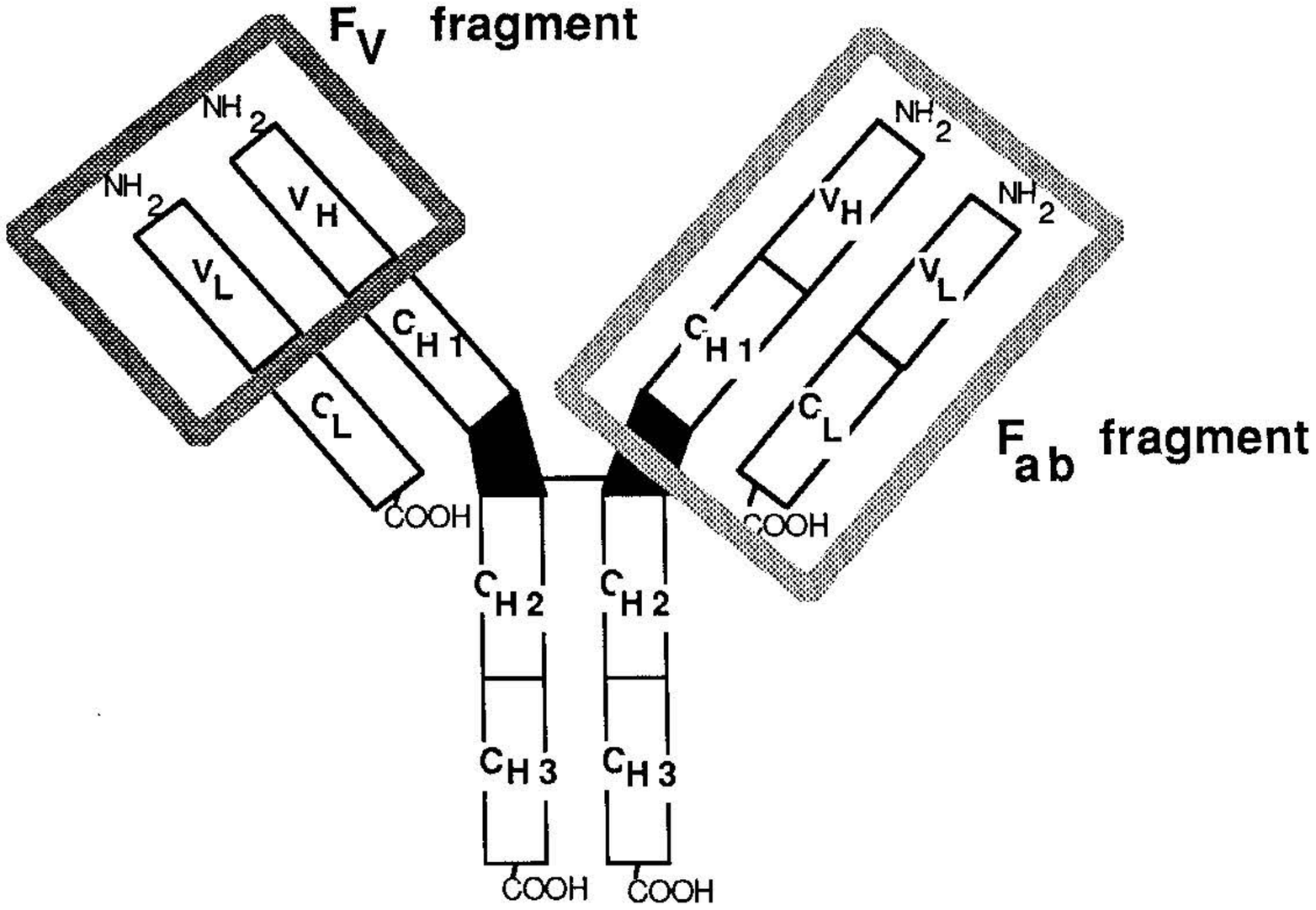

Fig. 1. Schematic representation of antigen binding fragments of an antibody

First, the production of altered antibody fragments is greatly simplified. The gene technology of *E. coli* is well established, and one can make genetic constructions directly in the expression vectors. The production is much faster (because of the fast growth rate of *E. coli*), and very much simpler (because of the comparatively simple cultivation of *E. coli*). Biotechnological optimization of antibody production in *E. coli* is just beginning, and will probably soon provide very inexpensive routes to recombinant antibodies.

Second, one may now directly produce fragments only consisting of the binding site such as the F_v or F_{ab} fragment (Fig. 1), as well as fusions to other proteins such as marker enzymes, toxins or metal binding fragments, which are of interest in tumor imaging and therapy. While a bacterial expression system is not needed for producing such fusion proteins, it greatly facilitates the work.

Third, the high transformation rate of *E. coli* permits for the first time the establishment of libraries. This introduces the great potential of bacterial genetics into antibody production, be it in the form of random mutagenesis (to improve a binding constant, or to eliminate a cross-reactivity) or in the form of cloning naturally occuring antibodies from the repertoire. This bacterial expression technology makes it now conceivable that one day antibodies will be available only by screening of well designed libraries without resorting to animals. Such libraries might then also be created from human sources.

In this article, I will review this technology of the expression of antibody fragments in *E. coli*, and discuss the properties of several of the resulting fragments.

Expression

The technology we developed is based on secreting both chains making up the antibody fragment to the periplasmic space of *E. coli*. In this way, they are transported to an oxidizing milieu, and either chain can therefore act as the folding template for the other. With this secretion technology, both chains making up the F_{ab} fragment (i.e. V_HC_H [the F_d fragment] and V_LC_L [the complete light chain]) ore those making up the F_v fragment (i.e. just V_L and V_H) (Fig. 1) can be co-secreted and the corresponding fragments are functionally assembled [3, 4]. There is a number of critical steps in the expression and secretion pathway that must be designed correctly for the F_v and F_{ab} fragment to assemble into functional fragments, and vectors designed for this strategy have been described [3, 4, 16].

(i) Stoichiometric amounts of both chains must be synthesized. Our strategy for accomplishing this goal was to construct an artificial operon, in which both genes are under the control of the same promotor and both coding regions are preceded by Shine-Dalgano sequences.

(ii) Both chains must be transported to the periplasm, using fusions to *E. coli* signal sequences. While we have found that a variety of signal sequences will function on both chains (A. SKERRA and A. PLÜCKTHUN, unpublished) we have used in most of the work the signal sequence from the outer membrane protein A (*ompA*) for one chain, and the signal from alkaline phosphatase for the other chain (*phoA*).

(iii) Both signal sequences must be processed at the correct position to yield the identical N-terminus as the original antibody from the animal or human. The location of cleavage was verified experimentally and found to be identical as in the natural antibody.

(iv) In the periplasmic space, the two chains must then fold to globular domains, with a simultaneous oxidation of the intramolecular disulfide bonds, and the two chains must assemble. While the in vivo mechanism of this process is not yet clear, and the participation of *E. coli* proteins is currently being investigated in our laboratory, we can clearly state that this assembly process does take place in vivo in the periplasm of *E. coli*.

The secretory strategy has a number of advantages. First, it directly leads to assembled functional fragments with correctly formed disulfide bonds, and there is no need to refold the protein in vitro. Second, the problem of proteolytic degradation is greatly diminished, as there are far fewer proteases in the periplasm than in the cytoplasm. The folded globular domains are also more resistant to proteases as they do contain the disulfide bonds. This would not be possible in the cytoplasm. The correct in vivo assembly then allows the purification of the fragment directly by its antigen binding affinities using antigen affinity chromatography (summarized in Fig. 2). Alternatively, we have recently designed a new one-step purification independent of the antigen-binding properties, based on immobilized metal ion affinity chromatography [16].

Our original experiments were carried out with the especially well characterized antibody McPC603 as a model system. McPC603 is a phosphorylcholine binding IgA of the mouse. This protein was originally obtained as a myeloma protein and has been extensively characterized [17]. The sequence, the crystal structure of its F_{ab} fragment with and without bound antigen [18, 19], as well as binding constants and binding kinetics of several haptens have been determined [17], facilitating the characterization of the recombinant products. We had originally obtained the genes for the variable domains synthetically and also linked them to appropriate constant domains to encode the F_{ab} fragment [20]. The recombinant technology has now been used with this model system to study antigen binding [21], catalysis by the antibody [22, 23] and the influence of mutations on the three-dimensional structure [24].

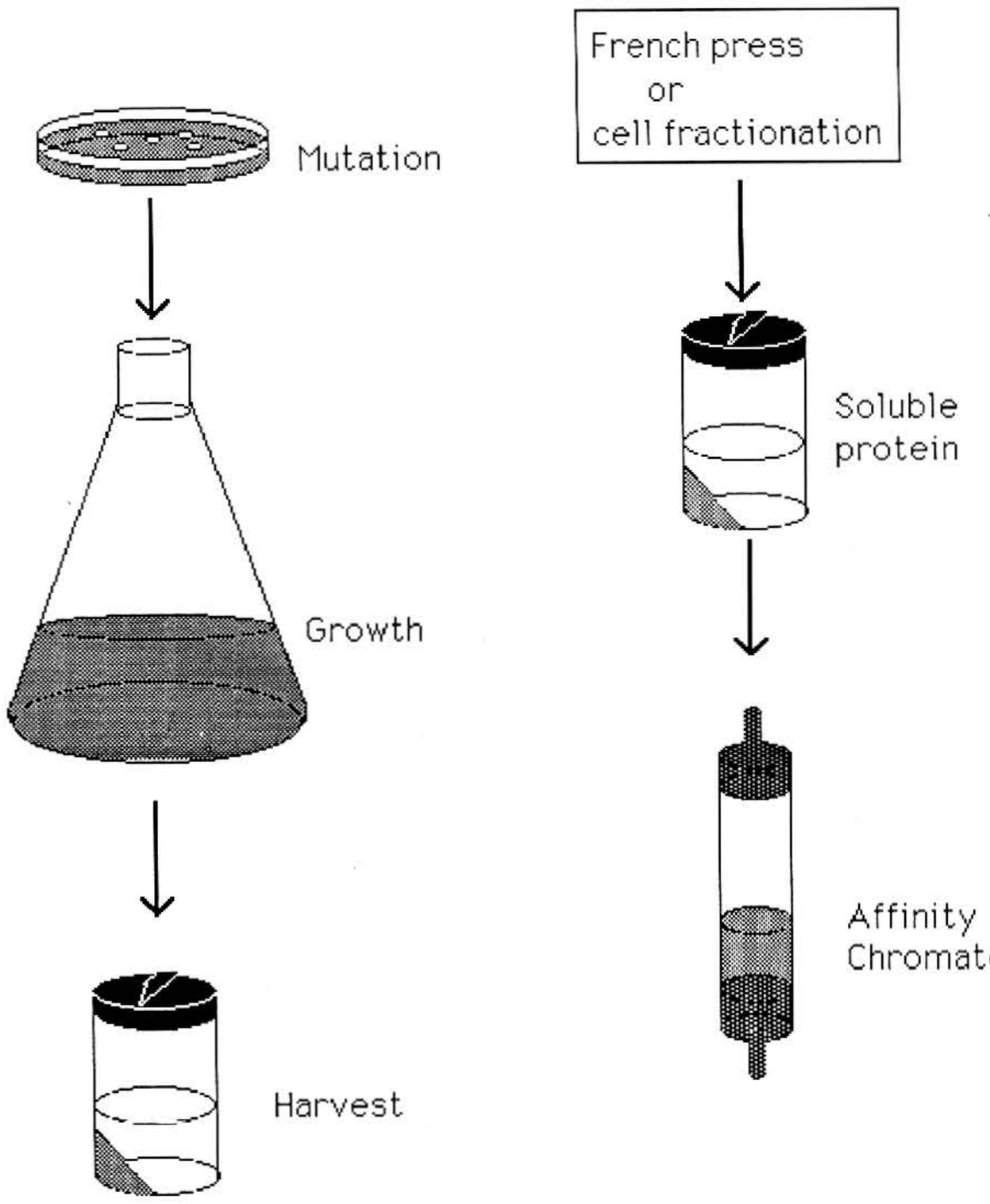

Fig. 2. Production of fully functional antibody fragments in *E. coli* on the laboratory scale. After the desired change or mutation has been made, the cells are grown in Erlenmeyer flasks, centrifuged and either lysed completely with a French press [3, 4, 15] or the periplasmic fraction is prepared [16]. The insoluble material is separated by centrifugation and the soluble material can be directly applied to an antigen affinity column.

Properties of Various Antibody Fragments Expressed in *E. coli*

The envisaged applications will largely determine the choice of the antibody fragment to be expressed. If antibodies are used for industrial or laboratory applications only, binding and stability are probably the main criteria. In this case, F_v fragments, single-chain F_v fragments and F_{ab} fragments (Fig. 1, Fig. 3) are probably good choices. If antibodies are intended for human use, however, it will have to be decided, whether the natural effector functions of the whole antibody are desired or whether instead artificial effector functions should be spliced onto much smaller fragments of the antibody. I will now discuss some of the properties of the various antibody fragments using the mouse IgA McPC603 as an example. From this antibody, we have obtained a large number of fragments all containing the same antigen binding site.

F_{ab} Fragments

F_{ab} fragments of antibodies can be easily prepared by proteolytic cleavage and have therefore been well characterized. They usually have the same antigen binding activities as the whole antibody [25]. It was shown for the F_{ab} fragment of McPC603 that it has exactly the same binding constant whether expressed in *E. coli* or obtained by proteolysis from the mouse

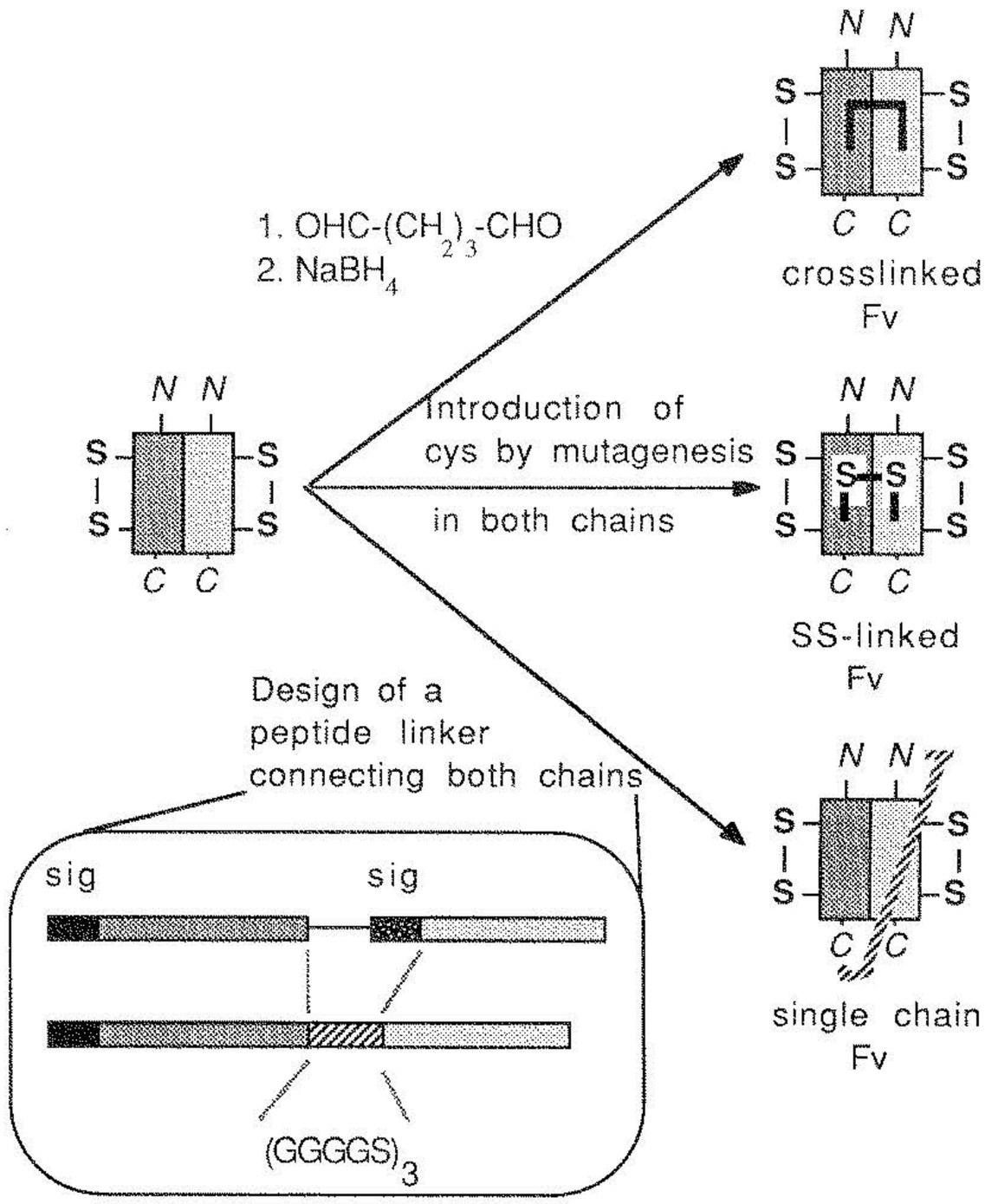

Fig. 3. Strategies for covalently linking F_V fragments
The top reaction describes a purely chemical strategy, namely crosslinking with glutaraldehyde with subsequent reduction of the Schiff bases. The middle arrow describes the introduction of one cysteine residue in each chain by mutagenesis to allow the formation of an intramolecular disulfide bond in each chain. The bottom arrow describes the linking of the two domains by a genetically encoded peptide linker. On the gene level, the intergenic region and the downstream signal must be replaced by the sequence coding for the peptide linker. In this case [15], the sequence (gly-gly-gly-gly-ser)$_3$ was used as the linker.

antibody [26]. Even the fact that the C_H1 domain is glycosylated in this mouse IgA has no effect on the antigen binding constant. In antibodies of the IgG class, the antibody F_{ab} fragment is not glycosylated, and therefore identical molecules can be obtained in *E. coli* as in the mouse. The constant domains increase the stability of the fragment and protect it against dissociation of the two chains. On the other hand, we have found (A. SKERRA and A. PLÜCKTHUN, unpublished) that the folding efficiency of the F_{ab} fragment is somewhat lower than that of the F_v fragment. The exact molecular reasons for this difference are currently under investigation.

F_v Fragments and Single-Chain F_v Fragments

F_v fragments are the smallest antibody fragments that still contain the complete antigen binding site. They are difficult and often impossible to prepare by proteolysis and therefore rather little had been known about their properties before they became available through gene technology [3]. It has been shown for the F_v fragment of McPC603 [3, 15] that it does have the same binding properties as the F_{ab} fragment or the whole antibody. It was also noted, however, that some F_v fragments have a tendency to dissociate into V_H and V_L upon

dilution [15]. The exact equilibrium constants for domain dissociation will vary from antibody to antibody, since the hypervariable loops contribute to this interaction. For McPC603, the dissociation constant was found to be about 10^{-6} M [15].
An important application for F_v fragments may be in tumor diagnostics and therapy, since the small size may allow the penetration of dense tumor tissue and provide for low antigenicity. Yet, much work remains to be done about tumor localization and the in vivo pharmacokinetic properties of these fragments, as they have become available only very recently. The small size of the F_v fragment makes it also a particularly interesting target for structural studies such as X-ray crystallography and NMR [24, 27].
The problem of dissociation of F_v fragments into V_H and V_L can be counteracted by three strategies [15] (Fig. 3). The first approach is to chemically crosslink the two chains with glutaraldehyde. In the case of McPC603 this is accomplished without any loss of binding affinity. The crosslinked species have been used in studies to determine the quantitative contributions of single amino acids in antigen binding interactions using the methods of site directed mutagenesis [21]. The second strategy is to design an intermolecular disulfide bond. The oxidized species with the additional disulfide bond between the two domains form spontaneously in the periplasm of *E. coli*, and the molecule can therefore be obtained in fully functional form from *E. coli*. Again, antigen binding affinity was only very marginally affected in the case of McPC603 [15]. The third strategy is to connect the two domains by a peptide linker. The resulting fragment has been called single-chain F_v fragment. Such peptide linked single-chain F_v fragments had previously been obtained as insoluble inclusion bodies and have to be refolded in vitro [28, 29] but the single-chain F_v fragment can also be obtained in fully functional form by secretion [15]. Again, the affinities were almost identical to the complete antibody [15].

Isolated Domains

The properties of isolated domains will again vary from antibody to antibody. It is possible that in some cases antigen binding will be observed, whereas in other cases it will not be possible. We have investigated the properties of isolated V_H and V_L domains of the antibody McPC603. The V_H domain is only poorly soluble at temperatures about 4 °C and is unable to bind the antigen. Therefore, the use of V_H domains as a general substitute for antibody combining sites [30] faces some problems. Especially the selectivity, which is one of the hallmarks of antibodies, can only be maintained in a binding site consisting of all six hypervariable domains.
Isolated V_L domains may dimerize. The dissociation constant will vary depending on the specific antibody, and usually lies in the micromolar to millimolar range. The V_L domain of McPC603 does not bind the antigen either, but this recombinant domain was recently shown to give very well ordered crystalls of the dimer [24]. The structure was determined and it now offers the exciting prospect of rapidly obtaining a structural database of characteristic complementarity determining regions using protein engineering. Fortunately, many variants crystallize isomorphously (B. STEIPE, R. HUBER, and A. PLÜCKTHUN unpublished).

Downstream Processing and Purification

In addition to the conventional techniques of protein purification, there are two additional techniques especially suitable for the purification of antibody fragments from *E. coli* expression systems. The first one is antigen affinity chromatography. Since the antigen binding properties of the expressed antibodies will be known beforehand, this technique

can be applied rationally. We have shown in the case of McPC603 that the molecule can be purified from a crude lysate of *E. coli* in one single step [3, 4, 15].
More recently, we have developed a more general purification technique that took advantage of protein engineering. The C-terminus of the variable domains was altered to contain a stretch of consecutive histidine residues which are able to bind to a metal ion immobilized on a column (Immobilized Metal Ion Chromatography, IMAC) [16]. With this technique, fragments can be purified to homogeneity in a single step independent of their properties, and this opens up exciting prospects of producing other proteins of the immunoglobulin superfamily using similar strategies.

Challenges for Biotechnology

The production of antibodies in bacteria has now reached a point, where several problems of the basic science have been solved, but the procedures leave room for technical optimization in many aspects. For instance, production yields could be improved further by developing very high density fermentation processes of *E. coli* suitable for secretion. This would make the process economically highly interesting as a large scale production method, apart from its obvious biotechnological importance as a tool in antibody engineering. In contrast, very expensive nutrients as used in the production of labeled antibody fragments for NMR experiments will require the optimization of product yield per biomass, even at the expense of obtaining inclusion bodies.
Biochemical factors contributed by the host strain and their importance for the expression process have not yet been fully understood, and the role of membrane stability, proteases and other yet undiscovered factors is still not clear. Further optimization will also have to include the study of folding modulators (molecular chaparones) as in at least some fragments the folding process itself seems to be limiting (SKERRA and PLÜCKTHUN, unpublished).
The screening and production of antibody fragments in *E. coli* will become a key technology in the design, selection and preparation of antibodies for technical and medical use. An interdisciplinary approach of protein engineering, biotechnology and immunology will be required to mount a true challenge to the mouse and rabbit as designers and producers of antibodies.

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