Comparison of the Fv Fragments of Different Phosphorylcholine Binding Antibodies Expressed in *Escherichia coli*

ANDREAS PLÜCKTHUN AND ILSE PFITZINGER

*Genzentrum der Universität München*

c/o Max-Planck-Institut für Biochemie

Am Klopferspitz

D-8033 Martinsried, Germany

The production of antigen-binding fragments of antibodies in *Escherichia coli* greatly facilitates further research on antibodies, their engineering, and numerous applications in biotechnology and medicine. We previously developed a system with which fully functional Fv fragments (the heterodimer of the \(V_H\) and \(V_L\) domain) and Fab fragments (the heterodimer of the whole light chain \([V_LC_L]\) and the first two domains of the heavy chain \([V_HC_H]\)) can be expressed in *E. coli.*

The purification can be carried out in a single step by hapten affinity chromatography. Our strategy consists of the simultaneous expression and secretion of both chains of the antibody fragment into the periplasm of *E. coli.* Considerations in the development of this approach were that both chains must fold simultaneously and in each other’s presence, and that the folding and assembly must take place in the oxidizing environment of the periplasm. The cytoplasm, where protein synthesis occurs, has a reducing environment, and we have shown that neither the Fv nor the Fab fragment can be obtained in functional form without the correct formation of the disulfide bonds in the variable domains. Thus, the transport of the two chains to the periplasm is a prerequisite for obtaining fully functional antigen-binding fragments.

Previous experiments were carried out with the antibody McPC603 (or M603), an IgA of the mouse that binds phosphorylcholine. Its particularly attractive features include the known three-dimensional structure and substantial previous work on its binding properties. In this article, we describe experiments comparing this antibody with the related antibody TEPC15 (or T15), both expressed as Fv fragments in *E. coli.* These antibodies, together with MOPC167 (or M167), are the three “prototypes” of the murine immune response after challenge with phosphorylcholine. A mutational analysis of M167 will be described elsewhere (Schweder and Plückthun, in preparation).

The Fv fragment is the smallest conceivable fragment that has the complete binding pocket of an antibody. We previously showed that the intrinsic binding constant of the Fv fragment is almost identical to that of the whole antibody. Therefore, the presence of the constant domains has no influence on the structure of the antigen-binding site. However, the association energy between the \(V_L\) and the \(V_H\) domain is not very high, and the Fv fragment dissociates into these domains at high dilution. This leads to complicated binding behavior, which can be deconvoluted into an intrinsic binding constant identical to the whole antibody and a finite association constant of the two domains. The association constant between \(V_L\) and \(V_H\) depends on the precise structure of the antibody and therefore indirectly on its specificity, because the hypervariable loops contribute several important interactions.
for the association of the two domains to form the Fv fragment. Furthermore, the antigen itself contributes to the stabilization of the Fv fragment.\textsuperscript{11}

This dissociation of the Fv fragment into V\textsubscript{H} and V\textsubscript{L} can be prevented by either of three strategies\textsuperscript{11}: first, chemical cross-linking of the two domains; second, the introduction of disulfide bonds; or third, the construction of a peptide linker to give a so-called single-chain Fv fragment\textsuperscript{11-13} We could show that such a single-chain Fv fragment can still be transported and that it folds correctly inside the periplasm.\textsuperscript{11} Its can also be purified by hapten affinity chromatography, and the measured hapten binding affinity is very similar to that of the whole antibody. Interestingly, however, the amount of functional fragment is not increased by the peptide linker. Thus, the mutual finding and association of the two domains in the periplasm of \textit{E. coli} do not seem to constitute a problem.

Another strategy to stabilize the heterodimer is to express the F\textsubscript{ab} fragment\textsuperscript{24,14,15} which contains constant domains C\textsubscript{L} and C\textsubscript{H1} in addition to the variable domains. The recombinant F\textsubscript{ab} fragment gave exactly the same binding constant as did the whole antibody or the F\textsubscript{ab} fragment obtained by proteolysis of the whole antibody.\textsuperscript{15} We also found that the proteolytic F\textsubscript{ab} fragment differs from the recombinant one by glycosylation in the C\textsubscript{H1} domain.\textsuperscript{15} This identity of binding constants between the recombinant and the proteolytic material clearly demonstrates that this glycosylation has no influence on binding. Because the hapten binding constants of the F\textsubscript{ab} fragment and the whole antibody are also identical, the presence of the constant domains C\textsubscript{H2} and C\textsubscript{H3} (and any glycosylation therein\textsuperscript{16}) has no influence on antigen binding either.

We did observe, however, that the yield of functional F\textsubscript{ab} fragment is consistently smaller than that of the functional F fragment expressed in \textit{E. coli}. A detailed analysis of this phenomenon\textsuperscript{4} showed that the problem is not one of expression or secretion, but one of folding and assembly.

Other investigators have attempted to cut down the size of the F\textsubscript{v} fragment even further by using only V\textsubscript{H} as a binding domain.\textsuperscript{17} Our investigations of single domains have shown, however, that the V\textsubscript{H} domain is difficult to handle because of limited solubility (Glockshuber and Plückthun, unpublished experiments). The V\textsubscript{L} domain, on the other hand, is very soluble and has a tendency to dimerize. We have not obtained any evidence for binding the phosphorylcholine antigen by either domain alone. The recombinant V\textsubscript{L} domain of M603 produced in \textit{E. coli} could recently be crystallized, and its crystal structure was solved to a resolution of 2.0 Å. It has now been refined to an R factor of 14\%\textsuperscript{18,19}

We have now extended this expression strategy to the family of the other phosphorylcholine binding antibodies and corresponding mutants. This allows the use of protein-engineering experiments combined with structural and mechanistic studies to compare their binding, stability, folding, and catalytic properties. We report in this paper the gene synthesis and expression of the F\textsubscript{v} fragment of the antibody T15 and its comparison with M603. The detailed examination of well-characterized antibodies is the prerequisite for the development of efficient screening and selection procedures in \textit{E. coli}, and the family of phosphorylcholine binding antibodies is well suited for such experiments.

**MATERIALS AND METHODS**

\textit{Recombinant DNA Techniques and Protein Expression}

Recombinant DNA techniques were based on those of Maniatis \textit{et al.}\textsuperscript{20} The antibody fragments were expressed in \textit{E. coli} JM83, using a vector\textsuperscript{21} with an \textit{f1} phage
origin\textsuperscript{22} and a resident repressor gene, a transcriptional terminator, which can therefore be used in many strains. In some cases, the \textit{ompT}\textsuperscript{-} strain UT4400 (ref. 23) was also used. Site-directed mutagenesis was carried out according to Kunkel \textit{et al.}\textsuperscript{24} and Geisselsoder \textit{et al.}\textsuperscript{25}

\textit{Gene Synthesis}

The oligonucleotides were synthesized with an Applied Biosystems Model 380A synthesizer. They were then purified by polyacrylamide gel electrophoresis, phosphorylated with polynucleotide kinase, hybridized, and ligated with T4 ligase using the same methodology as that in the synthesis of the genes of M603.\textsuperscript{14}

The operon of the T15 antibody\textsuperscript{26} was assembled as follows. A mutagenesis with four oligonucleotides of the expression plasmid encoding M603 (ref. 21) was carried out. Two of them introduced the required mutations in \textit{VH}, one introduced a silent change in the signal of \textit{VL}, creating a unique \textit{StyI} site in the plasmid, and one converted the COOH-terminal sequence of the \textit{VL} gene from M603 to T15. The piece between the \textit{StyI} and the \textit{BspMII} site was assembled from six oligonucleotides (three for each strand) and first subcloned into pUC19 by having extended the synthetic region to make it compatible with polylinker cloning sites. From this pUC19 derivative, the relevant piece was obtained as a \textit{StyI-BspMII} fragment and ligated to the previously mutagenized expression plasmid, cut with the same enzymes. The complete sequence of the operon was verified in the expression plasmid by DNA sequencing (Fig. 1).

\textit{Protein Purification}

Both recombinant antibody fragments were purified by phosphorylcholine affinity chromatography essentially as described previously\textsuperscript{1} except that the bacterial growth was performed at 20$^\circ$C and the cells were induced for 3 hours before the harvest.\textsuperscript{11} The cells were then disrupted in a French pressure cell, and the soluble part of the lysate was directly applied onto the affinity column.

\textit{Cross-Linking}

The cross-linking of the F\textit{_c} fragment with glutaraldehyde was carried out as described previously.\textsuperscript{11} The cross-linked F\textit{_c} fragment was then repurified by affinity chromatography.

\textit{Hapten Binding}

Hapten binding was followed by recording changes in protein fluorescence as described previously.\textsuperscript{11}

\textbf{RESULTS}

The response of the immune system to phosphorylcholine-containing antigens has been studied intensively.\textsuperscript{27} The natural immunogen is the surface polysaccharide
FIGURE 1. Sequence of the synthetic operon of T15. The signal sequences are underlined and stop codons denoted with a star. The relevant restriction sites as well as the junctions of the synthetic oligonucleotides are labeled. The beginning of the mature parts of VH and VL is also labeled.
of *Streptococcus pneumoniae* and several other microorganisms, which carries phosphorylcholine esterified on a sugar residue. The murine immune response results in the production of antibodies, all derived from the same V\_H gene, and this family of antibodies is named after the antibody carrying the genomic sequence T15. In contrast, three different V\_L genes are predominantly employed by the mouse. The resulting antibodies can be represented by myeloma protein "prototypes" McPC603 (or M603), TEPC15 (or T15), and MOPC167 (or M167). These myeloma proteins are of the IgA class and are the best studied examples of the phosphorylcholine binding antibodies. We have thus chosen these particular proteins as model systems. The same three types of antibodies are also elicited if the immunization is carried out with phosphorylcholine-derivatized protein antigen, but IgM and IgG are usually obtained in this case.

To be able to carry out comparative studies on structure, folding, binding, and antibody-mediated catalysis, we wished to express the F\_v fragments of all three antibodies (M603, T15, and M167) in *E. coli*. We had previously developed the *E. coli* expression technology with the antibody M603. An alignment of the variable domains V\_L and V\_H for all three antibodies is shown in FIGURE 2. We used leucine at position L112 (numbered according to the crystal structure of M603; also numbered 112 in Fig. 2) in the original synthesis of the light chain gene of M603 for homology reasons, although isoleucine was reported from

---

**FIGURE 2.** Comparison of the sequences of the V\_H and V\_L domains of M603, M167, and T15. In the sequences, deletions are marked with a dash. In the line below the three sequences, a star refers to an identity across all three sequences and a period refers to a conservative exchange. Underlined residues were not determined in the original publications, but obtained from homologies to similar antibodies. The hypervariable regions according to the definition of Kabat *et al.* are boxed and labeled.
more recent sequencing experiments (Rudikoff, unpublished work; quoted in ref. 5).
It is very unlikely that this substitution is of any significance as both residues are
commonly found in antibodies at this position. The light chain sequence for T15 was
not completely determined (summarized in ref. 26), but all residues of the sequence
that were determined are identical to the sequence of the antibody S107 (ref. 26),
and the missing residues were thus taken from the sequence of S107. The sequences
shown in Figure 2 then correspond to the domains used in the gene synthesis.

The heavy chains of the three myeloma proteins are rather similar, because they
are probably derived from the same \( V_H \) gene (reviewed in ref. 27). We could
therefore take advantage of the existing synthetic \( V_H \) gene of M603\(^{14} \) and obtain
the T15 \( V_H \) gene by site-directed mutagenesis with several oligonucleotides simulta­
neously (see the Materials and Methods section). This mutagenesis was carried
out directly in the expression vector for the \( F_v \) fragment that is described else­
where.\(^{21} \) On this vector, the two genes encoding \( V_H \) and \( V_L \) are arranged in a synthetic
operon under the control of one regulatable promoter.\(^{12,21} \)

The light chains, on the other hand, are sufficiently different so that such a
mutagenesis strategy would not be advantageous. We therefore decided to com­
pletely synthesize the light chain of T15, analogous to the previous synthesis of the
genes for M603. The synthetic strategy took advantage of restriction sites present in
the synthetic operon of M603,\(^{14} \) so that a synthetic cassette of T15 could replace the

The phosphorylcholine binding \( F_v \) fragments of M603 and T15 can be purified by
hapten affinity chromatography in a single step, directly demonstrating the function­
ality of the \( F_v \) fragments produced in \( E. coli \). Interestingly, however, the amounts of
functional fragment isolated were lower for T15 (about 10–20%), although vectors,
translation imitation regions, signal sequences, strains, and experimental conditions
were identical. Codon use in the synthetic genes is essentially identical in the two
operons, and rare codons\(^{11} \) have largely been avoided in both constructs. No
differences in possible hairpins are apparent in the two genes. The expression of the
plasmid encoding T15 was also examined in a host strain deficient in the protease

![FIGURE 3. SDS PAGE of the Fv fragments of M603 and T15 expressed in E. coli and purified by hapten affinity chromatography. A 15% polyacrylamide gel stained with Coomassie brilliant blue is shown. The upper band corresponds to VH (122 amino acids for M603 and 123 amino acids for T15); the lower one corresponds to VL (115 amino acids for both M603 and T15).](image_url)
FIGURE 4. Scatchard plot of the binding of phosphorylcholine to the recombinant Fv fragment of the antibody T15. \( r \) denotes the fraction of the antibody with a bound ligand and \([PC]\) the molar concentration of free hapten.

OmpT,\(^{23,32}\) but no influence on the level of expression of functional T15 Fv fragment was found compared to that of an ompT+ host (data not shown). Most likely, proteolytic sensitivity to an as yet unidentified protease causes this difference in expression level, and this effect is even more pronounced for M167 (Pfitzinger, Schweder, and Plückthun, unpublished observations).

We then examined the binding constant of the Fv fragment of T15. The dissociation of the Fv fragment at low concentration requires cross-linking of the \( V_H \) and \( V_L \) domains for fluorescence measurements\(^{11} \) or high protein concentrations for equilibrium dialysis measurements\(^{1} \) to obtain linear Scatchard plots with binding constants identical to the Fab fragment or the whole antibody. Both experimental approaches have been shown to be in quantitative agreement.\(^{11} \) This demonstrates that modification of surface lysines by glutaraldehyde with subsequent reduction to obtain the cross-linked Fv fragment, which preserves the positive charge, does not affect the binding of the hapten. We therefore employed fluorescence measurements of the glutaraldehyde cross-linked Fv fragments to determine hapten binding constants (Fig. 4).

The association constant obtained for the Fv fragment of T15 (3.3 × 10^5 M\(^{-1}\)) is in good agreement with binding constants previously reported for the whole antibody\(^{6,8,33} \) (2.3 × 10^5 M\(^{-1}\) to 5.5 × 10^5 M\(^{-1}\)). Antibody T15 thus binds PC slightly better than does antibody M603, and this trend is seen in both the Fv fragments and the whole antibody, validating the recombinant methodology.

DISCUSSION

The three antibodies, M603, M167, and T15, are "prototypes" of the three classes of antibodies that are generated when a mouse is challenged with the antigen phosphorylcholine. Their heavy chains are derived from the same \( V_H \) gene, but each one uses a light chain derived from a different \( V_L \) gene.\(^{27} \) This particular system of related antibodies now allows the investigation of several mechanistic questions of folding, binding, and antibody-mediated catalysis by the use of protein engineering.

The first aspect of interest is the structure of these three antibodies. Only the structure of the Fab fragment of McPC603 has been experimentally determined,\(^{3} \) but for the structure of the other two, only models have so far been proposed.\(^{34} \) The main problem in modeling these related structures is that some loops forming part of the binding pocket are not equal in length (Fig. 5). This difference occurs mainly in the CDR3 loop of the heavy chain, which is partially derived from the D gene. It may also differ in length because of the so-called N-region diversity.\(^{27} \) Thus, a large number of
degrees of freedom is introduced into modeling the active site by the uncertainty of the structure of these loops. The availability of the recombinant Fv fragments will now allow a renewed attempt at structure determination by both NMR and X-ray crystallography. This is of particular importance for the critical evaluation of the various modeling attempts and for obtaining feedback about the quality of different theoretical approaches.

The second aspect that may be analyzed with these recombinant antibodies is the functional importance of different somatic mutations that have accumulated and constitute differences to the germ-line genes. The recombinant Fv fragments can now be used to delineate which of these mutations were selected to secure chain association or antigen binding, and which of these mutations are due to random drift without particular benefit to the function of the molecule. This question also requires an examination of the question, which combinations of heavy and light chains are able to associate in vivo and function in phosphorylcholine binding.35

The third aspect that can now be scrutinized is that of catalysis. Previous results with the antibodies M167 and T15 obtained from mouse ascites28,29 and with the recombinant Fv fragment of M603 and some of its mutants (ref. 30; Stadlmüller and Plückthun, manuscript in preparation) have shown that these antibodies have catalytic activity in hydrolyzing carbonate esters of choline. The activities reported
for M167 and T15 are higher than those for M603, but the mechanistic and structural reasons have not been elucidated. These mechanistic questions can now be addressed experimentally by protein engineering of the Fv fragments.

SUMMARY

The development of general methods to express functional antibody fragments in E. coli greatly facilitates the engineering of antibodies. Some of the essential features of the technology are summarized. As a model system, phosphorylcholine binding antibodies are used. The immune response against this antigen results in three classes of antibodies, exemplified by the myeloma proteins McPC603, TEPC15, and MOPC167. Fv fragments of these antibodies can now be conveniently prepared in E. coli to aid in understanding the structural logic of this well-characterized immune response.

REFERENCES


