[34] Expression of Functional Antibody Fv and Fab Fragments in *Escherichia coli*

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General Considerations

Introduction

The highly selective binding properties of antibodies make these proteins important reagents in research, medical diagnostics, and therapy. Numerous sophisticated procedures have been developed to obtain antisera with narrow and predetermined specificity,[1] but a decisive breakthrough was only achieved with the discovery of methods to produce monoclonal antibodies[2] (see this series, Vols. 73, 92, and 121). Besides their defined specificity, monoclonal antibodies offer the advantages of invariant properties and the potential to be produced in large quantities. The advent of gene technology now makes it possible to go one step further: to produce antibodies not present in the natural pool. By modifying the DNA sequence coding for it, the antibody molecule itself can be altered.

While techniques to change DNA sequences are firmly established (site-directed mutagenesis: see, e.g., this series, Vol. 154; gene synthesis[3]), the expression of antibodies is not. In this chapter, we describe procedures and experiences with a very convenient expression system, namely, the expression of *functional* Fv and Fab fragments of antibodies in *Escherichia coli*.

At the present time, fine-tuning the specificity of an antibody by protein engineering in a rational and predetermined way is still in a very early stage. However, the investigation of the relationship between sequence, structure, binding properties, and stability of an antibody itself depends on a convenient expression system, such as the *E. coli* expression system for native antibody fragments described here.

Antibodies are part of the large immunoglobulin superfamily.[4] Many of the molecules responsible for the cellular immune response are structurally related to antibodies. It is probable, therefore, that many results valid

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[1] D. M. Weir, L. A. Herzenberg, C. Blackwell, and L. A. Herzenberg, "Handbook of Experimental Immunology," 4th ed., Vol. 1. Blackwell, Oxford, 1986.

[2] G. Köhler and C. Milstein, *Nature (London)* **256**, 495 (1975).

[3] J. Engels and E. Uhlmann, *Adv. Biochem. Eng. Biotechnol.* **37**, 73 (1988).

[4] A. F. Williams and A. N. Barclay, *Annu. Rev. Immunol.* **6**, 381 (1988).

for an antibody expression system will be applicable to other molecules of the superfamily. Thus, detailed structural investigations of, e.g., the T-cell receptor, the major histocompatibility complex, or other cell interaction molecules may become easier.

Engineered Antibodies and Antigen-Binding Fragments for Different Applications

The potential to alter the antibody molecule itself has opened up a number of intriguing possibilities. Antibodies for diagnostic or research purposes might be developed that bind to variants of an antigen which are normally not recognized or that bind to poorly immunogenic molecules. Moreover, one might engineer antibodies that do *not* bind to molecules which cause a background problem in a particular assay.

The use of cloned genes also permits an alteration in the framework and the constant domains of an antibody. Each case will have to be evaluated as to whether the minimal active antigen-binding fragment (the Fv fragment) or the Fab fragment is advantageous for a particular application. For engineering the antigen-binding properties of an antibody, Fv fragments should usually be sufficient. The altered Fv fragment may then still be fused to appropriate constant domains for a specific application. Constant domains and framework regions might be recruited from different species, as was reported, e.g., in experiments describing the "humanization" of antibodies.[5-7]

The construction of completely new hybrid molecules that do not occur in nature should also be feasible with an approach based on our expression system. For instance, fusions of antigen-binding fragments with reporter enzymes may be constructed to facilitate the development of immunodiagnostic reagents. Metal-binding domains might be used for the detection of antibodies via the binding of radioactive metal ions (e.g., in tumor imaging[8]). The cross-linking of antibodies to toxins has attracted much attention[8,9]; a convenient expression system for genetically encoded protein fusions may facilitate further developments in this field. An important consideration in this context is that the toxin should not be toxic to the host cell that produces the fusion protein. Bacteria may,

[5] P. T. Jones, P. H. Dear, J. Foote, M. S. Neuberger, and G. Winter, *Nature (London)* **321**, 522 (1986).

[6] L. Riechmann, M. Clark, H. Waldmann, and G. Winter, *Nature (London)* **332**, 323 (1988).

[7] M. Verhoeyen and L. Riechmann, *BioEssays* **8**, 74 (1988).

[8] H. H. Sedlacek, G. Schulz, A. Steinstraesser, L. Kuhlmann, A. Schwarz, L. Seidel, G. Seemann, H.-P. Kraemer, and K. Bosslet, *in* "Contributions to Oncology," Vol. 32. Karger-Verlag, Berlin, 1988.

[9] G. Möller, ed., "Antibody Carriers of Drugs and Toxins in Tumor Therapy," Immunol. Rev., Vol. 62. Munksgaard, Copenhagen, 1982.

therefore, offer advantages over eukaryotes for fusions directed, for example, against human carcinoma cells.

A convenient expression system may also benefit the field of catalytic antibodies.[10-13] With a knowledge of the three-dimensional structure of the substrate–antigen complex, protein engineering may be used to investigate and improve catalytic properties of antibodies.[13] Furthermore, random mutagenesis and screening procedures may be envisaged that complement the rational protein-design approach.

While the rational modification of protein properties is the distant aim of protein engineering, an improvement of molecular properties (e.g., binding or catalysis) by a screening approach may be a more immediately reachable goal in many cases. Screening may even be carried out with no knowledge of the three-dimensional structure. The crucial point is, however, that for any screening procedure, the expression system must produce a *functional* antibody fragment. Moreover, it should permit the convenient manipulation of a large number of clones, which is most easily achieved in a bacterial expression system.

Structure of Antibodies and Antigen-Binding Fragments

The monomeric antibody molecule can be proteolytically cleaved into two identical antigen-binding fragments (Fab) and one constant fragment (Fc). The Fab fragment contains the complete light chain (with the variable domain V_L and the constant domain C_L) and the first two domains (V_H and C_H1) of the heavy chain.

All binding interactions to the hapten or antigen occur within the variable domains (V_H and V_L). Several years ago, a method for producing fragments containing only V_H and V_L (called Fv) by proteolysis was reported,[14] but the procedure is technically difficult. For this proteolytically obtained Fv fragment[14] and for a recombinant Fv fragment produced in *E. coli*,[15] it was shown that the affinity constants of the Fv fragments were indistinguishable from those of the corresponding whole antibodies.

The Fv fragment is the minimal structure for antigen-binding activity. It contains two chains of about 115 amino acids, which is of the size where structure and interactions can still be determined by NMR techniques.

[10] J. Jacobs, P. G. Schultz, R. Sugasawara, and M. Powell, *J. Am. Chem. Soc.* **109**, 2174 (1987).

[11] S. J. Pollack, J. W. Jacobs, and P. G. Schultz, *Science* **234**, 1570 (1986).

[12] A. Tramontano, K. D. Janda, and R. A. Lerner, *Science* **234**, 1566 (1986).

[13] A. Plückthun, R. Glockshuber, I. Pfitzinger, A. Skerra, and J. Stadlmüller, *Cold Spring Harbor Symp. Quant. Biol.* **52**, 105 (1987).

[14] D. Inbar, J. Hochman, and D. Givol, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2659 (1972).

[15] A. Skerra and A. Plückthun, *Science* **240**, 1038 (1988).

Also, the Fv fragment may offer advantages in cases where a small molecular size and low antigenicity of the molecule are crucial or where the presence of the constant domains may interfere with an experiment. Since, until now, there was no convenient method of producing Fv fragments, almost nothing is known about their *in vivo* properties and pharmacokinetics.[8] The Fab fragment, while also being monovalent for antigen recognition, might be more stable at very high dilutions.

Several three-dimensional structures of Fab fragments have been determined.[16-19] Structures of complexes consisting of Fab fragments and protein antigens have now been solved as well.[20-23] Each antibody domain consists of a sandwich of β sheets (with a layer of four and three strands making up the constant domains and layers of four and five strands forming the variable domains). The functional groups interacting with the antigen are supplied by amino acids from the hypervariable loops, which connect the β strands in the variable domains. Each variable domain has three such hypervariable regions [also referred to as complementarity-determining regions (CDR)].

As a model system, we chose the antigen-binding fragments (Fv and Fab) of an antibody that is very well characterized, namely, the phosphorylcholine-binding immunoglobulin A McPC603 of the mouse. The protein sequence,[24,25] three-dimensional structure,[26,27] hapten binding constants, and binding kinetics[28-33] have been determined for this

[16] P. M. Alzari, M.-B. Lascombe, and R. J. Poljak, *Annu. Rev. Immunol.* **6**, 555 (1988).
[17] D. R. Davies and H. Metzger, *Annu. Rev. Immunol.* **1**, 87 (1983).
[18] E. A. Kabat, *Adv. Protein Chem.* **32**, 1 (1978).
[19] M. Marquart and J. Deisenhofer, *Immunol. Today* **3**, 160 (1982).
[20] A. G. Amit, R. A. Mariuzza, S. E. V. Phillips, and R. J. Poljak, *Science* **233**, 747 (1986).
[21] P. M. Colman, W. G. Laver, J. N. Varghese, A. T. Baker, P. A. Tulloch, G. M. Air, and R. G. Webster, *Nature (London)* **326**, 358 (1987).
[22] R. Huber and W. S. Bennett, *Nature (London)* **326**, 334 (1987).
[23] S. Sheriff, E. W. Silverton, E. A. Padlan, G. H. Cohen, S. J. Smith-Gill, B. C. Finzel, and D. R. Davies, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8075 (1987).
[24] S. Rudikoff and M. Potter, *Biochemistry* **13**, 4033 (1974).
[25] S. Rudikoff, Y. Satow, E. Padlan, D. Davies, and M. Potter, *Mol. Immunol.* **18**, 705 (1981).
[26] Y. Satow, G. H. Cohen, E. A. Padlan, and D. R. Davies, *J. Mol. Biol.* **190**, 593 (1986).
[27] D. M. Segal, E. A. Padlan, G. H. Cohen, S. Rudikoff, M. Potter, and D. R. Davies, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4298 (1974).
[28] M. A. Leon and N. M. Young, *Biochemistry* **10**, 1424 (1971).
[29] H. Metzger, B. Chesebro, N. M. Hadler, J. Lee, and N. Otchin, in "Progress in Immunology" (C. B. Amos, ed.), p. 253. Academic Press, New York, 1971.
[30] N. M. Young and M. A. Leon, *Immunochemistry* **14**, 757 (1977).
[31] A. M. Goetze and J. H. Richards, *Biochemistry* **16**, 228 (1977).
[32] A. M. Goetze and J. H. Richards, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2109 (1977).
[33] A. M. Goetze and J. H. Richards, *Biochemistry* **17**, 1733 (1978).

myeloma protein. Various related antibodies[34-36] have also been partially characterized. Thus, a thorough characterization of the heterologously expressed molecules could be carried out, which is essential for demonstrating that the *E. coli* expression system leads to fully functional molecules.

Figure 1 shows an α-carbon tracing of the Fab fragment based on the coordinates determined by Davies and co-workers.[26,27] The Fv part (V_H and V_L) is emphasized by different shading. The arrangement of the disulfide bonds in the Fab fragment of McPC603 is schematically shown in Fig. 2. In the Fv fragment, there is one disulfide bond in each domain, connecting Cys-L23 to Cys-L94 in V_L and Cys-H22 to Cys-H98 in V_H. In the complete light chain of the Fab fragment, there is one additional disulfide bond in the constant domain, connecting Cys-L140 with Cys-L200. Furthermore, the carboxy-terminal amino acid L220 is a cysteine, which is partially free and partially bound to the other *light* chain Cys-L220 in mouse immunoglobulin A[37] and thus free in the monomeric Fab fragment. In the heavy chain C_H1 domain, there is the characteristic central intradomain disulfide bond connecting Cys-H148 with Cys-H206 and an additional disulfide bond, typical[37] for IgA, connecting Cys-H198 with Cys-H222. In most antibody classes, an intermolecular H–L disulfide bond is formed between Cys-L220 and a cysteine around H140; however, there is no other cysteine in McPC603. Thus, there is no intermolecular disulfide bond in either the Fv or the Fab fragment of McPC603.

The genes of the two variable domains V_H and V_L of McPC603 were synthesized as described elsewhere.[13] The genes encoding the Fd fragment of the heavy chain (amino acids H1–H222) and the complete κ light chain (amino acids L1–L220) were obtained by fusing the synthetic genes of the variable domains to cloned genes of the C_H1 domain or the C_L domain, respectively.[13]

Requirements for Antibody Expression Systems

There are two entirely different requirements for a practical expression system of recombinant antibodies or antibody fragments. First, it should be convenient to use, and, second, it should yield functional antibodies or antigen-binding fragments.

[34] E. A. Kabat, T. T. Wu, M. Reid-Miller, H. M. Perry, and K. S. Gottesmann, "Sequences of Proteins of Immunological Interest," 4th ed. U.S. Dept. of Health and Human Services, Public Health Serv., Natl. Inst. Health, Bethesda, Maryland, 1987.
[35] R. M. Perlmutter, S. T. Crews, R. Douglas, G. Sorensen, N. Johnson, N. Nivera, P. J. Gearhart, and L. Hood, *Adv. Immunol.* 35, 1 (1984).
[36] M. Potter, *Adv. Immunol.* 25, 141 (1977).
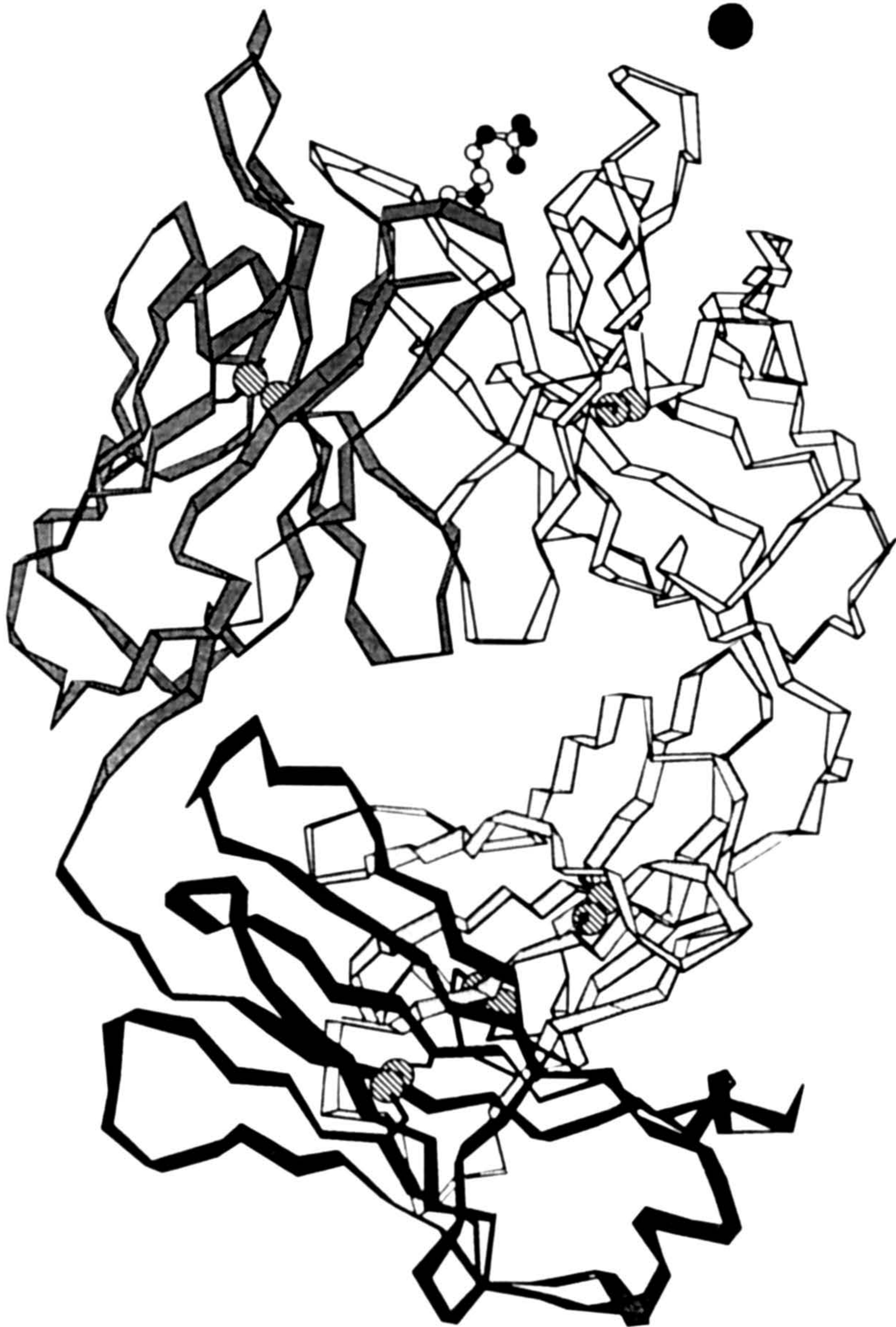[37] S. A. Cockle and N. M. Young, *Biochem. J.* 225, 113 (1985).

FIG. 1. Ribbon diagram of the Fab fragment of McPC603. The coordinates of Davies and co-workers[26,27] were used with the plot program of Lesk and Hardman [A. M. Lesk and K. D. Hardman, *Science* **216,** 539 (1982)]. The α-carbon tracing of the light chain is shown on the left and colored in gray (for V_L, top) and dark gray (for C_L, bottom). That of the heavy chain is shown on the right in white (for V_H, top) and light gray (for C_H1, bottom). The domain boundaries are colored according to the actual lengths of the variable domains V_L and V_H used in the recombinant Fv fragment. The sulfur atoms of the disulfide bonds are shown as hatched circles. The hapten phosphorylcholine is colored according to atom types with O in black, P in light gray, C in white, and N in darker gray.
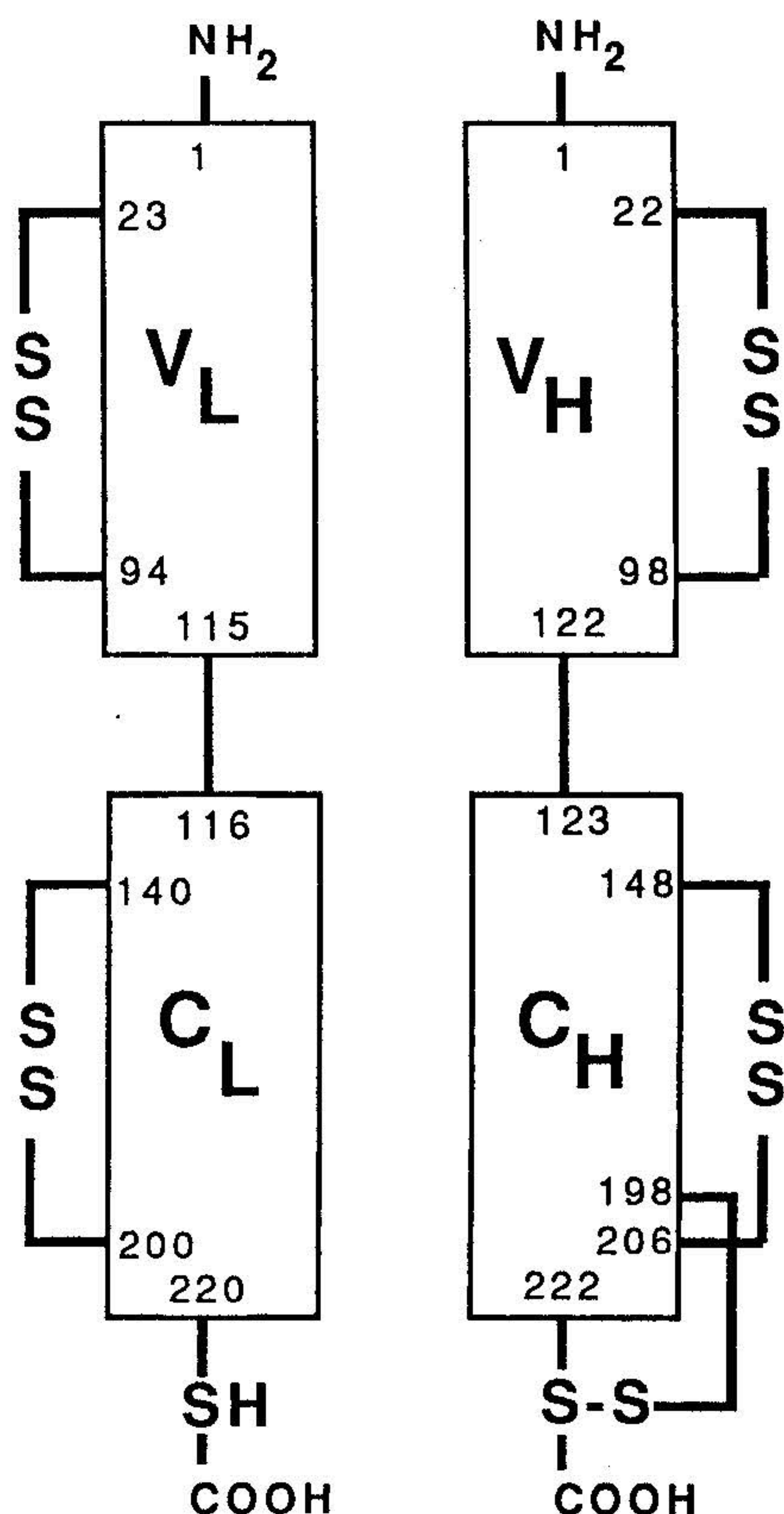
FIG. 2. Schematic drawing of the disulfide bonds in the Fab fragment of McPC603. Note the absence of any intermolecular disulfide bonds. The residue numbers of all the cysteines are indicated, as well as the domain boundaries used in the recombinant Fv fragment.

A microbial system, such as *E. coli*, is most advantageous for keeping laboratory procedures simple. The transformation of *E. coli* is very efficient, allowing one not only to directly transform the expression host with small amounts of DNA from genetic constructions but also to transform with pools of *in vitro* mutagenized DNA for the generation of libraries that can be screened for random mutants. Much is known about the genetics and biochemistry of *E. coli* so that sophisticated genetic experiments, rational strain improvement, or biochemical screening and selection procedures may be carried out. There are many genetic markers as well as selectable features, such as antibiotic resistances, that are known which may be useful in designing screening methods for antibodies with certain binding or catalytic properties. The kinetics of growth for *E. coli* is fast. Few problems involving sterility in fermentation are encountered, and

experimentation can be rather rapid. The large-scale fermentation of *E. coli* is also quite straightforward, and the isolation of large quantities of the antibody-combining fragments should be feasible. Some of the advantages of using *E. coli* are shared by other microbial expression hosts, e.g., *Bacillus subtilis* and yeast. However, the larger body of knowledge about *E. coli* makes the development of expression procedures for this bacterium simpler.

The second requirement for a good expression system is that the antibodies or antigen-binding domains be expressed in a functional state. The purification of the antibody fragment can make use of the antigen- or hapten-binding property itself. The use of an affinity column makes purification very selective and rapid. Also, the antigen-binding properties of the antibody will usually be known beforehand, whereas the properties of each individual combining fragment on various columns may not be, so that the development of a purification procedure is facilitated by an expression in the native state. All experiments that involve random mutants will need to be carried out with an expression system for functional molecules, since the individual refolding of a large number of mutants is clearly not feasible.

Simultaneously with others,[15,38] we have reported a procedure by which native antigen-binding fragments of antibodies can be produced by *E. coli,* thus combining the advantages of bacterial expression with those of expression in the native state. The expression and secretion of functional antibodies and Fab fragments have now also been achieved in yeast,[39] albeit with lower yields than with the *E. coli* expression system described in this chapter.

Several groups[6,7,40–44] have investigated the expression of recombinant antibodies in cells of higher eukaryotes. For producing a particular, well-defined antibody, eukaryotic expression systems may be attractive. This is true if the whole antibody is required for proper glycosylation, for instance, in human therapy. For applications in which the whole antibody molecule is not desired or in which rapid access to different engineered antibody molecules is essential, a bacterial expression system will be superior.

[38] M. Better, C. P. Chang, R. R. Robinson, and A. H. Horwitz, *Science* **240**, 1041 (1988).
[39] A. H. Horwitz, C. P. Chang, M. Better, K. E. Hellstrom, and R. R. Robinson, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8678 (1988).
[40] S. L. Morrison, *Science* **229**, 1202 (1985).
[41] S. L. Morrison and V. T. Oi, *Adv. Immunol.* **44**, 65 (1989).
[42] M. S. Neuberger, G. T. Williams, E. B. Mitchell, S. S. Jouhal, J. G. Flanagan, and T. H. Rabbitts, *Nature (London)* **314**, 268 (1985).
[43] M. S. Neuberger, G. T. Williams, and R. O. Fox, *Nature (London)* **312**, 604 (1984).
[44] S. Roberts, and A. R. Rees, *Protein Eng.* **1**, 59 (1986).

Different Expression Strategies in E. coli

For most applications, a purified and correctly folded antibody is essential. There are several criteria of correct folding and assembly that should be fulfilled by a recombinant antibody or antibody fragment obtained from any expression system: (1) the correctly folded and assembled antibody combining fragment must be easily purified, (2) the binding constant must be identical to the native antibody, (3) the stoichiometry of binding must be 1 : 1 (for monofunctional binding fragments), and (4) the S–S bonds must be formed correctly. Four methods have been described for expressing antibodies or binding fragments in *E. coli*.

1. The direct cytoplasmic expression of antibodies or antibody fragments has been reported previously.[13,45–48] The yields obtained were rather variable and critically depend on the design of the translation initiation signals and the choice of host strain. A simplified partial purification of the polypeptides by centrifugation requires that the expression is high enough for the protein to form inclusion bodies. The resulting polypeptides are denatured, reduced, and usually carry a methionine or formylmethionine at their amino terminus.[49] It is unclear whether this may affect the binding properties of an antibody. This expression method requires an efficient refolding procedure, a convenient method for the purification of the refolded and assembled antibody heterodimer, as well as stringent assays for the functionality and homogeneity of the preparations.

2. To overcome problems of inefficient translational initiation and proteolytic degradation, and to obtain a precise amino terminus, fusions with highly expressed cytoplasmic proteins can be constructed. We have fused[13,50] the V_H and V_L domains of McPC603 to β-galactosidase linked by a cleavage site for the rather specific protease of the blood-clotting cascade, factor Xa, and worked out refolding conditions for the cleaved domains.[50,51] A facile selective purification of the folded and assembled binding fragment through hapten-affinity chromatography was helpful in

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45 M. A. Boss, J. H. Kenten, C. R. Wood, and J. S. Emtage, *Nucleic Acids Res.* **12**, 3791 (1984).

46 M. A. Boss and C. R. Wood, *Immunol. Today* **6**, 12 (1985).

47 S. Cabilly, A. D. Riggs, H. Pande, J. E. Shively, W. E. Holmes, M. Rey, L. J. Perry, R. Wetzel, and H. L. Heyneker, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3273 (1984).

48 H. Field, A. R. Rees, and G. T. Yarranton, *in* "Vaccines 88" (H. Ginsberg, F. Brown, R. A. Lerner, and R. M. Chunock, eds.), p. 29. Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1988.

49 F. Sherman, J. W. Stewart, and S. Tsunasawa, *BioEssays* **3**, 27 (1985).

50 R. Glockshuber and A. Plückthun, unpublished.

51 J. Hochmann, M. Gavish, D. Inbar, and D. Givol, *Biochemistry* **15**, 2706 (1976).

establishing these conditions in the case of McPC603. For other antigen–antibody systems, working out a refolding procedure may be more complicated.

3. To overcome the refolding problem, fusions have also been constructed to the *Staphylococcus aureus* protein A,[13,52–54] a protein that is normally periplasmic or even secreted into the growth medium. This may conceivably permit the folding of the variable domains even as a hybrid protein. The advantage of this expression method is that the hybrid protein may be rapidly purified using an affinity column with IgG as the affinity ligand.[55] The method does, however, require an *in vitro* cleavage of the hybrid protein, either with a specific protease or by selective chemical cleavage.

4. In the fourth approach, which is described in detail in this chapter, we have designed an *E. coli* expression system *not* requiring any *in vitro* manipulation of the protein.[15] For this purpose we attempted to reproduce in *E. coli* the normal folding and assembly pathway of antibodies within the eukaryotic cell. The two chains are separately expressed as precursors with amino-terminal signal sequences and separately transported to the lumen of the endoplasmic reticulum (ER), where the signal sequences are cleaved. In the lumen of the ER, folding of the protein, S–S bond formation, and assembly of the light and heavy chains to the complete antibody occur.[56–59] In the lumen of the ER and the Golgi apparatus the heavy chain is also glycosylated, but this glycosylation is not required for binding activity.

The crucial hypothesis in the design of the latter expression system was that the protein transport into the periplasm of *E. coli* is functionally equivalent to the transport of a protein into the lumen of the ER. We therefore developed a system that directs both chains of the antibody-combining fragment to the periplasm of the same *E. coli* cell (Fig. 3). It is known that many heterologously expressed proteins fold correctly when transported to the periplasm of *E. coli*,[60] but the current expression sys-

[52] A. Plückthun and I. Pfitzinger, unpublished.

[53] L. Abrahmsen, T. Moks, B. Nilsson, and M. Uhlen, *Nucleic Acids Res.* **14**, 7487 (1986).

[54] L. Abrahmsen, T. Moks, B. Nilsson, U. Hellman, and M. Uhlen, *EMBO J.* **4**, 3901 (1985).

[55] T. Moks, L. Abrahmsen, B. Österlöf, S. Josephson, M. Östling, S. O. Enfors, I. Persson, B. Nilsson, and M. Uhlen, *BioTechnology* **5**, 379 (1987).

[56] L. Hendershot, D. Bole, and J. F. Kearney, *Immunol. Today* **8**, 111 (1987).

[57] S. Beychok, *in* "Cells of Immunoglobulin Synthesis" (B. Pernis and H. J. Vogel, eds.), p. 69. Academic Press, New York, 1979.

[58] R. Wall and M. Kuehl, *Annu. Rev. Immunol.* **1**, 393 (1983).

[59] M. D. Scharff, *Harvey Lect.* **69**, 125 (1975).

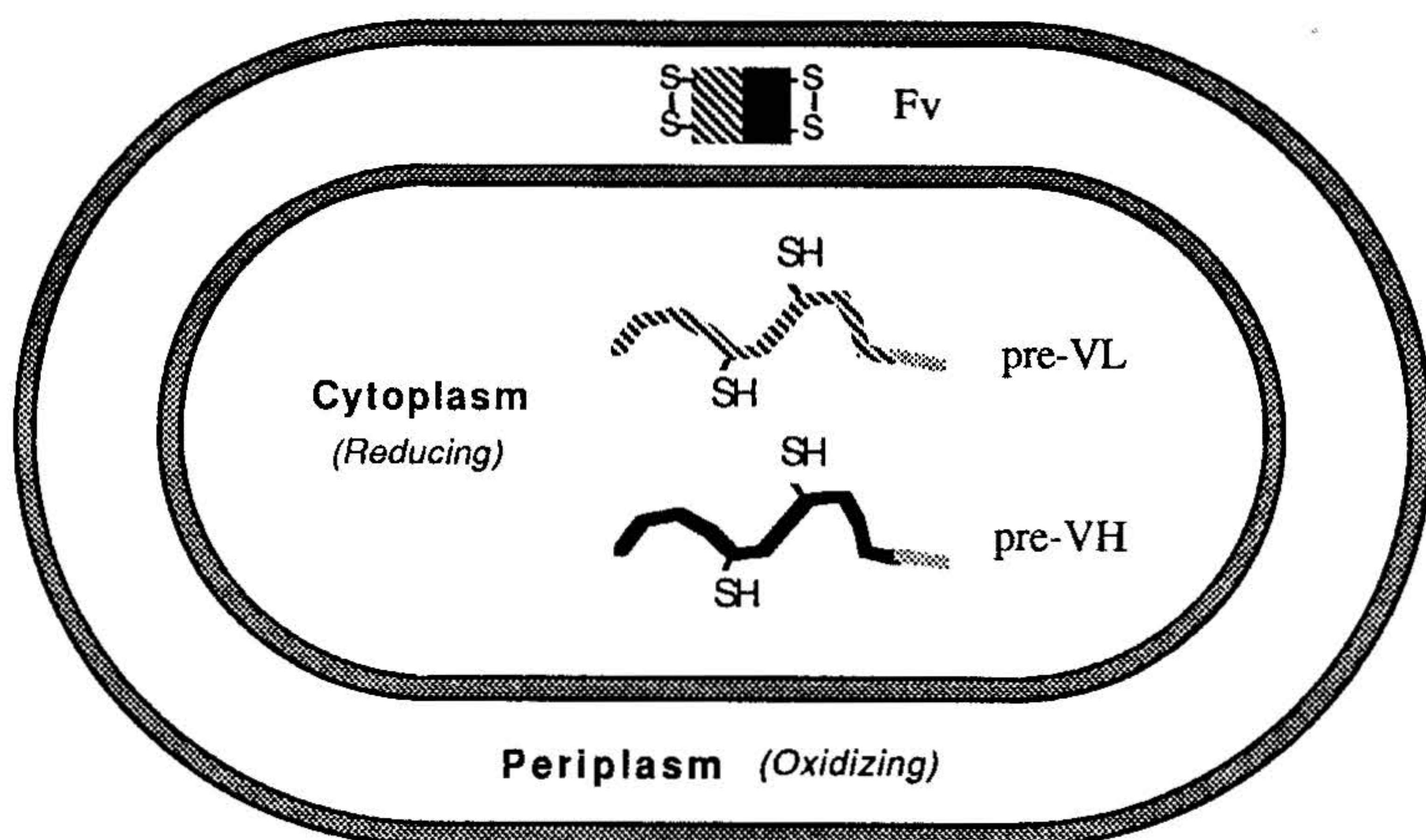[60] M. S. Briggs and L. M. Gierasch, *Adv. Protein Chem.* **38**, 109 (1986).

FIG. 3. Schematic diagram of the *E. coli* expression strategy for functional antigen-binding fragments. In the cytoplasm, the precursor proteins for V_L and V_H, each fused to a bacterial signal sequence, are synthesized in reduced form. After translocation through the inner membrane into the periplasm, the signal sequences are cleaved, the domains fold and assemble, and the disulfide bonds form (see text). Expression of the Fab fragment is completely analogous.

tem of an antibody-combining fragment may provide the first example in which the assembly of a heterodimer is required as well.

The main advantage of this expression system is that it directly leads to an assembled functional product with correct disulfide bonds. Another advantage of a periplasmic location is that the problem of protease degradation is much diminished. There are fewer proteases in the periplasm than in the cytoplasm, and folding to globular domains, accompanied by oxidation of the S–S bonds in the periplasm, may provide additional stabilization. Preparation of the periplasmic lysate is already an enrichment procedure for the recombinant protein and thus facilitates the purification since there are far fewer proteins in the periplasm than in the cytoplasm. Another consequence of the expression in the native state, as already mentioned above, is the simplified purification using the binding properties of the antibody itself.

Expression System for Functional Fv and Fab Fragments in
    Escherichia coli

Vector Design

There are several critical steps in expression and secretion that must occur correctly for the Fv or Fab fragment to assemble. Vectors that

fulfill all the requirements are shown in Fig. 4. (1) Approximately stoichiometric amounts of both chains must be synthesized. This is accomplished by designing an artificial operon, in which both genes are under the control of the same promoter and both coding regions are preceded by a Shine–Dalgarno sequence. (2) Both chains must be transported to the periplasm. This is achieved by precise fusion to two different *E. coli* signal sequences, one from the outer membrane protein A (*ompA*)[61] and the other from alkaline phosphatase (*phoA*).[62] (3) Both signal sequences must be cleaved off at the correct position to yield the identical amino termini as in the mouse myeloma protein. The precise fusion of the signal sequences is necessary but not always sufficient for this to occur, and the location of cleavage must be verified experimentally for both chains. This was done for the Fv fragment of McPC603 by automated Edman degradation,[15,63] and the correct cleavage was confirmed for both chains. (4) Folding to globular and soluble domains must occur; (5) the intramolecular disulfide bonds must form; and (6) the two chains must assemble to form a heterodimer. Both vector design and expression methodology influence these steps. The stoichiometry of expression is important in obtaining a high yield of heterodimer. The light chain can dimerize with itself, and the heavy chain may aggregate and precipitate under some conditions.[50]

The expression rate must be commensurate with the transport, folding, and assembly steps or the protein will accumulate as insoluble material. It is not useful, therefore, to employ extremely strong promoters. Also, the promoter must be silent in the uninduced state or problems with cell lysis may be encountered (see below). We use the *lac* promoter/operator[64] (Fig. 4). Since exposure to the oxidizing milieu of the periplasm during the folding process is necessary for the disulfide bonds to form, a vector secreting both chains is an essential requirement.

The correct assembly must again be proved experimentally; the critical test is determination of the binding constant and stoichiometry. In the case of the Fv fragment of McPC603, an affinity constant identical to the whole antibody could be demonstrated as well as a stoichiometry of binding of 1 mol hapten per mole Fv fragment.[15] This shows that the affinity-purified Fv fragment is fully functional. However, lower binding constants for a V_H domain–light chain heterodimer have been reported,[65] and it is not yet clear whether the full functionality of Fv fragments will be a general phenomenon. In the case of Fab fragments a full functionality

[61] N. R. Movva, K. Nakamura, and M. Inouye, *J. Biol. Chem.* **255,** 27 (1980).
[62] H. Inouye, W. Barnes, and J. Beckwith, *J. Bacteriol.* **149,** 434 (1982).
[63] C. Eckerskorn, W. Mewes, H. Goretzki, and F. Lottspeich, *Eur. J. Biochem.* **176,** 509 (1988).
[64] C. Yanish-Perron, J. Vieira, and J. Messing, *Gene* **33,** 103 (1985).
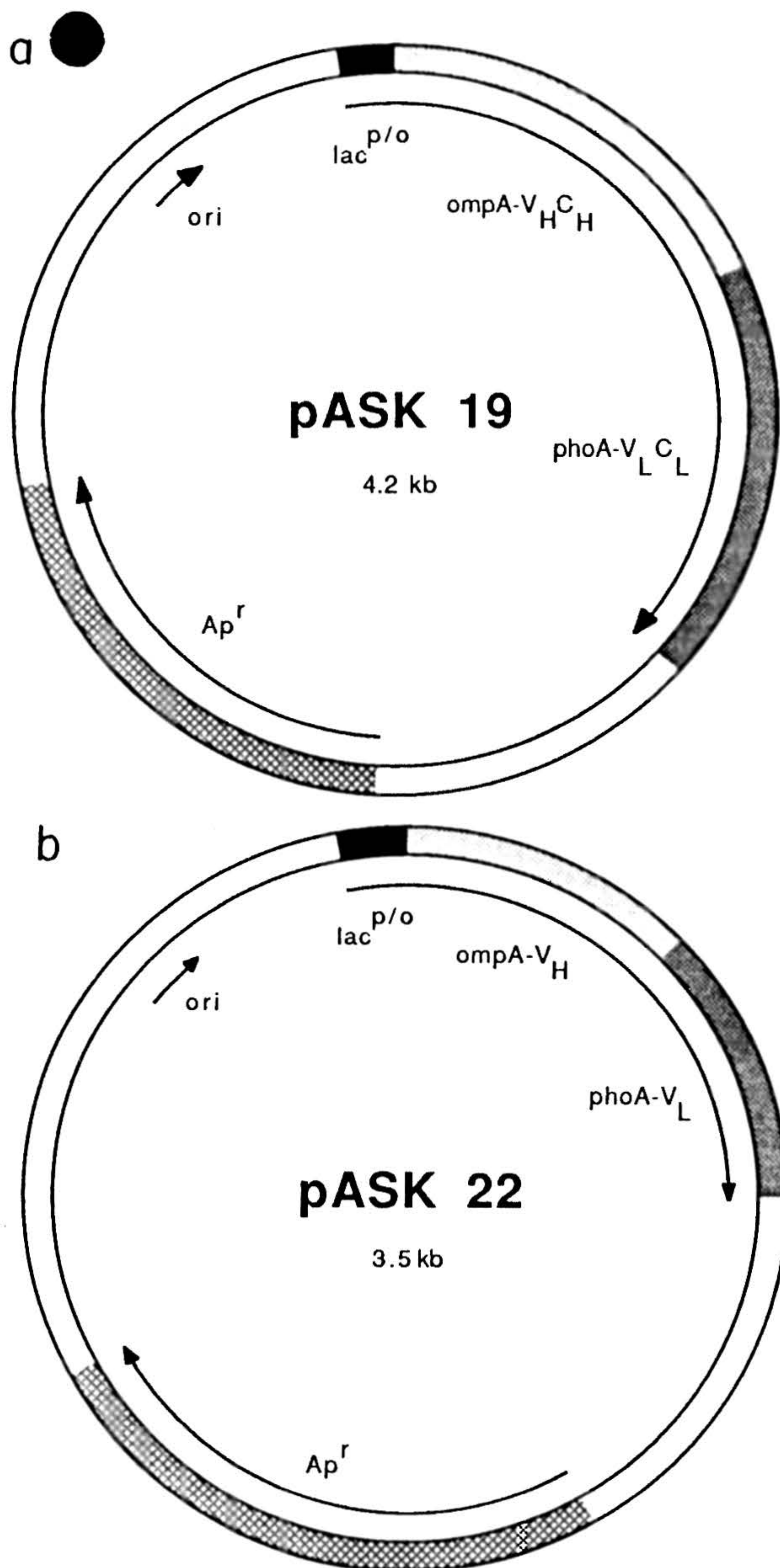[65] J. Sen and S. Beychok, *Proteins* **1,** 256 (1986).

FIG. 4. Plasmids pASK19 (a) and pASK22 (b) for the coexpression and cosecretion of the κ light chain ($V_L C_L$) and the Fd fragment ($V_H C_H$) or the V_L and V_H domains of McPC603, respectively. The plasmids carry the origin of replication (*ori*) and the ampicillin-resistance gene (*Ap^r*) from the pUC family of plasmids.[64] In pASK19, the $V_H C_H 1$ and the $V_L C_L$ domains are encoded on one transcription unit downstream of a *lac* promoter/operator[64] (*lac*^p/o) which is inducible by isopropyl-β-D-thiogalactoside (IPTG). The genes encoding the $V_H C_H 1$ and the $V_L C_L$ domains[13] are precisely fused to gene fragments encoding the signal sequence of outer membrane protein A (*ompA*)[61] and alkaline phosphatase (*phoA*),[62] respectively. Both genes are preceded by ribosomal binding sites to ensure efficient translation initiation. Plasmid pASK22 is identical to pASK19 except that the coding regions for the constant domains are absent.[15]

should normally be observed,[66] and Fv fragments should *usually* have the same binding properties as the corresponding whole antibody. Thus, determination of the affinity constant *and* stoichiometry of binding are the two crucial criteria in evaluating the expression for new antibody Fv or Fab fragments.

Expression Methodology

Protein Localization. In the expression system described here, the transport of both precursor proteins (V_H and V_L or $V_H C_H$ and $V_L C_L$) to the periplasmic space is desired. Leakage of periplasmic proteins into the culture medium is a phenomenon that is sometimes observed[67] when heterologous proteins are secreted in *E. coli*. For this reason, the operon was put under the control of an inducible promoter (*lac*).[64] Indeed, shortly after induction, the outer membrane of the cells begins to become permeable. This results in a liberation of periplasmic proteins, e.g., β-lactamase, into the growth medium and in simultaneous "leakage" of the Fv or the Fab fragment. In Fig. 5, a typical onset of lysis after induction is shown for the wild-type *E. coli* strain W3110, harboring plasmid pASK22, at 37°. This leakage is not a consequence of the expression of Fab versus Fv fragments nor of the choice of signal sequences, but it does depend on the strain and growth conditions.[67,68] In the procedure detailed here, leakiness of the cell is not desired, and a short induction time, suitable strain, and/or lower growth temperature are used to harvest the cells before lysis. In another report,[38] the periplasmic proteins were allowed to leak out into the growth medium by using a very long induction period. However, we find that purification from the small volume of pelleted cells is much more convenient than from the growth medium, which is contaminated with cell debris, and so we have worked out conditions for improving the yield of *periplasmically* localized Fv or Fab fragments.

Purification of Recombinant Fv and Fab Fragments. Purification of the Fv and Fab fragment involves only lysis of the cells, ultrafiltration, and antigen affinity column chromatography. Since the antibody-combining fragment is periplasmic and since the cells are fairly labile, the cells can be disrupted by a mild osmotic shock. This procedure releases only soluble periplasmic proteins, and the Fv or Fab fragment is therefore already considerably enriched. There are few proteases in the periplasm,

[66] S. J. Kennel, *J. Immunol. Methods* **55**, 1 (1982).
[67] I. Suominen, M. Karp, M. Lähde, A. Kopio, T. Glumoff, P. Meyer, and P. Mäntsälä, *Gene* **61**, 165 (1987).
[68] H. Takagi, Y. Morinaga, M. Tsuchiya, H. Ikemura, and M. Inouye, *BioTechnology* **6**, 948 (1988).
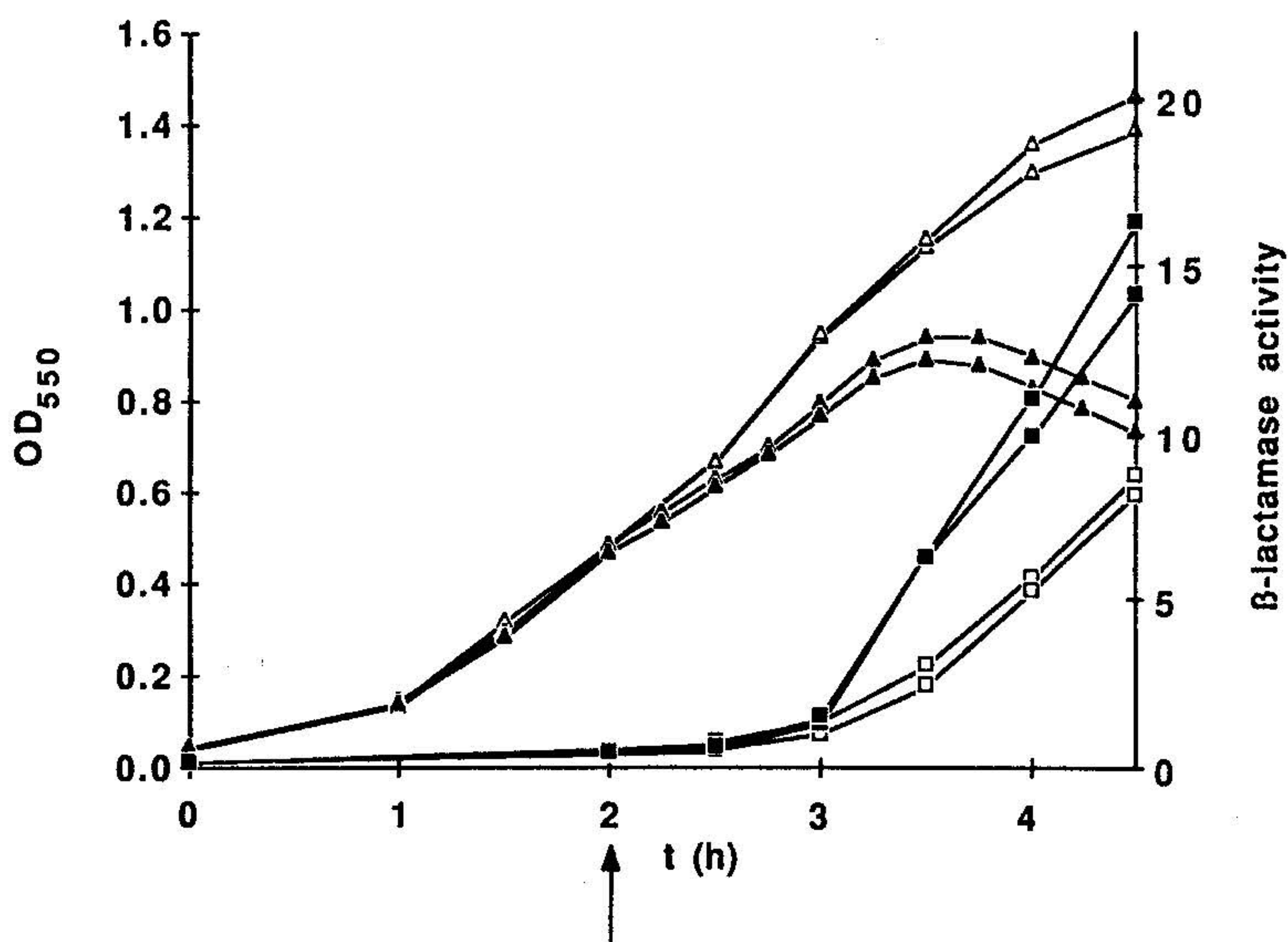
FIG. 5. Partial cell lysis after prolonged induction at 37°. Filled symbols refer to induced cultures and open symbols to uninduced control cultures. Experiments with duplicate cultures are shown. *Escherichia coli* strain W3110, harboring pASK22, was induced (arrow) by the addition of IPTG. Triangles indicate growth and lysis of the culture (measured as optical density at 550 nm), and squares indicate the activity of β-lactamase in the culture medium (arbitrary units).

and both Fv and Fab fragments are quite stable in the periplasmic lysate. The single purification step for recombinant fragments of McPC603 uses an antigen affinity column with a phosphate ester of phosphorylcholine as the ligand, and a selective elution with the hapten phosphorylcholine.[69] A typical elution profile is shown in Fig. 6.

The resulting purification to homogeneity is illustrated for the Fv fragment as well as the Fab fragment (Figs. 7 and 8). It can be seen from Fig. 7 that for the Fv fragment two bands with equally intense staining are obtained (corresponding to stoichiometric amounts of V_H with 122 amino acids and V_L with 115). The quality of the fractionation can be assessed from lanes 1 and 2. There are few bands in common between the periplasmic lysate (lane 2) and the total lysate (lane 1). Similar results are obtained for the Fab fragment (Fig. 8). Again, two bands of the expected size with equal intensity are present ($V_H C_H$ with 222 amino acids and $V_L C_L$ with 220). The recombinant light chain corresponds precisely to the natural

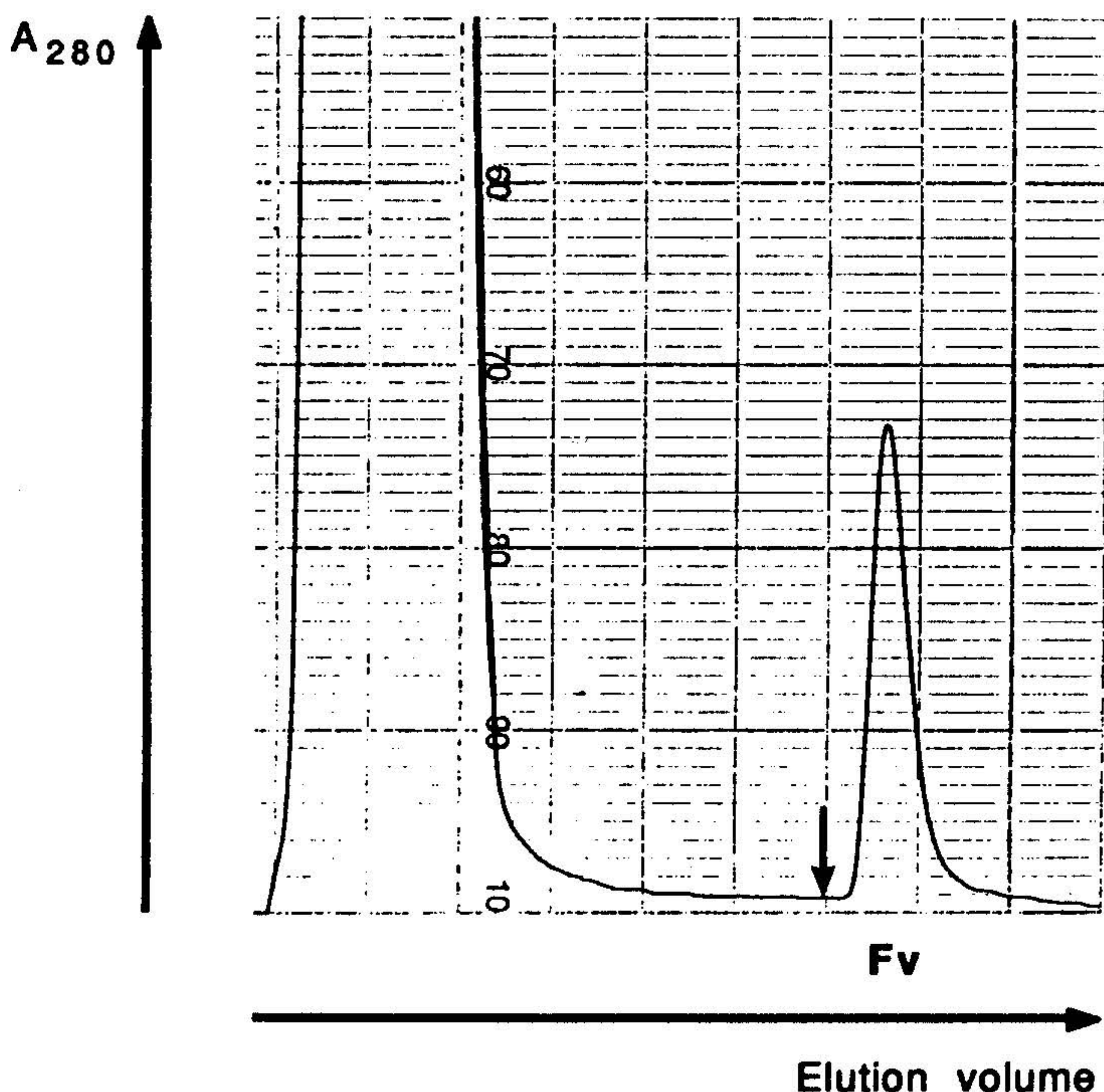[69] B. Chesebro and H. Metzger, *Biochemistry* **11**, 766 (1972).

FIG. 6. Affinity chromatography of the Fv fragment of McPC603. A typical elution profile from an affinity column with a phosphate ester[69] of phosphorylcholine as the ligand is shown. A periplasmic lysate (see text) was applied to the column. The column was washed with BBS buffer (see text) and eluted (arrow) with BBS buffer containing 1 mM phosphoryl-choline.

light chain of McPC603, and they comigrate on the gel as expected. The recombinant heavy chain ($V_H C_H$) is very slightly retarded compared to the light chain, and this is seen in gels from the direct cytoplasmic expression as well.[13] Interestingly, the percentage of correctly assembled protein is significantly lower for the Fab fragment than for the Fv fragment of McPC603, although the total amount of expressed heterologous protein is similar. We have not yet determined the causes of this difference.

Expression and Purification Method

For the expression of the Fv or Fab fragment of McPC603, a culture of *E. coli* strain W3110 harboring plasmid pASK22 or pASK19 is grown in
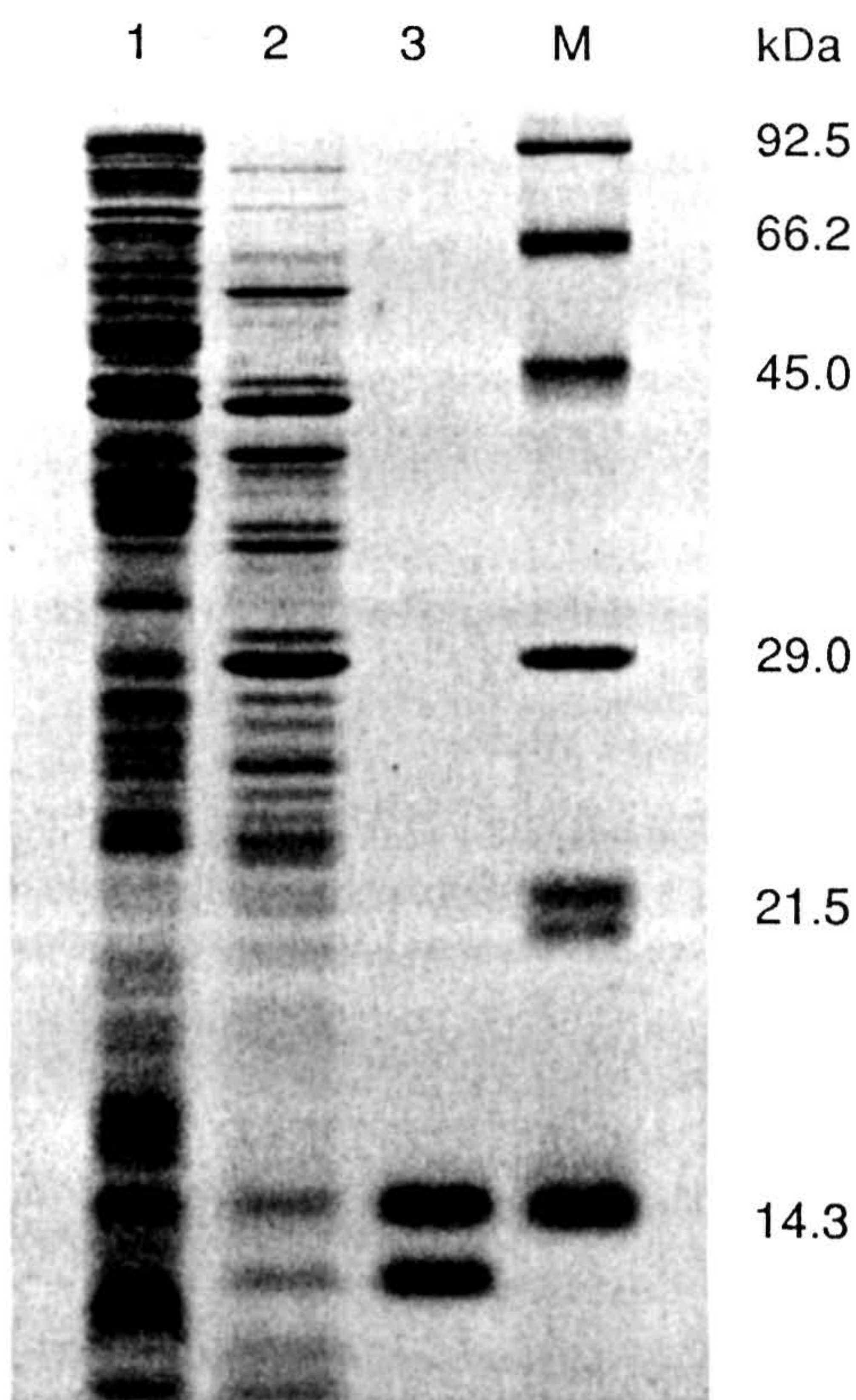
FIG. 7. Purification of the recombinant Fv fragment of McPC603 from *E. coli* W3110 harboring pASK22. A sodium dodecyl sulfate (SDS)–polyacrylamide (14%) gel stained with Coomassie Brilliant blue is shown. Lane 1, Total cell protein; lane 2, periplasmic fraction; lane 3, affinity-purified recombinant Fv fragment; lane M, protein size markers.

Luria-Bertani (LB) medium,[70] containing 100 mg/liter ampicillin, to an OD_{550} of 0.5. After induction for 45 min by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM, the cells are harvested by centrifugation at 4000 g for 10 min (at 4°). Cell fractionation is carried out by resuspending the cell pellets in TES buffer (0.2 M Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 M sucrose, 4°) (10 ml/liter of original culture). The cells are then subjected to a mild osmotic shock by addition of cold TES, diluted 1:4 with H_2O and containing 2 mM phosphorylcholine (15 ml/liter of original culture). After incubation on ice for 30 min, the

[70] J. H. Miller, "Experiments in Molecular Genetics." Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1972.
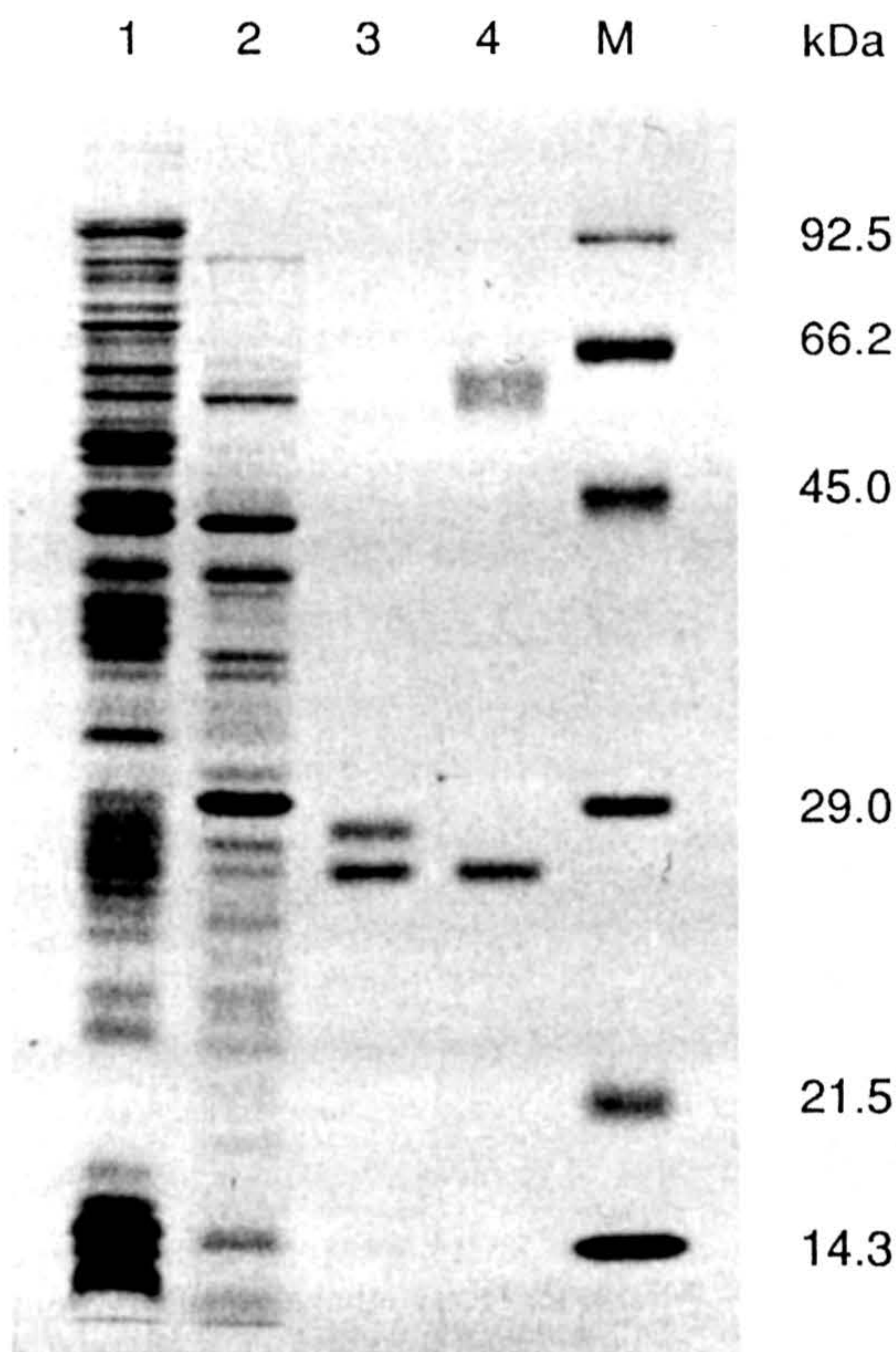
FIG. 8. Purification of the recombinant Fab fragment of McPC603 from *E. coli* W3110 harboring pASK19. An SDS–polyacrylamide (12.5%) gel stained with Coomassie Brilliant blue is shown. Lane 1, Total cell protein; lane 2, periplasmic fraction; lane 3, affinity-purified recombinant Fab fragment; lane 4, whole IgA McPC603, affinity-purified from mouse ascites; lane M, protein size markers.

suspension is centrifuged (5000 g, 10 min), and the supernatant is centrifuged again (48,000 g, 15 min). This final supernatant contains all soluble periplasmic proteins.

The quality of the cell fractionation can be monitored by using β-lactamase as a periplasmic and β-galactosidase as a cytoplasmic marker enzyme. For β-lactamase assays, nitrocefin[71] can be used as the substrate [0.2 mM in 50 mM phosphate buffer, pH 7.0; a stock solution of nitrocefin is made up in dimethyl sulfoxide (DMSO); measured at 486 nm]. For β-

[71] C. H. O'Callaghan, A. Morris, S. M. Kirby, and A. H. Shingler, *Antimicrob. Agents Chemother.* **1**, 283 (1972).

galactosidase, o-nitrophenylgalactoside is used as the substrate[70] [4 mg/ml in Z buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 50 mM 2-mercaptoethanol, pH 7.0); measured at 420 nm]. About 90% of the β-lactamase activity and less than 0.5% of the total β-galactosidase activity are found in the periplasmic fraction.

Using an Amicon YM5 or YM10 membrane, the final supernatant is concentrated by ultrafiltration to a volume of approximately 2.5 ml/liter of original culture and dialyzed repeatedly against BBS buffer (0.2 M borate-NaOH, pH 8.0, 0.16 M NaCl). This concentrated solution is applied to a phosphorylcholine affinity column[69] (2.5 ml bed volume per 4 liter of bacterial culture). After washing with BBS, pure Fv or Fab fragment is eluted with a solution of 1 mM phosphorylcholine in BBS. The typical yield under these conditions is approximately 0.2 mg purified Fv fragment per liter of bacterial culture, but the yield of the Fab fragment is lower. The total yield can be increased if the cell density at which induction is carried out can be increased without lysis, e.g., by different growth conditions.

Acknowledgments

[35] Recombinant Antibodies Possessing Novel Effector Functions

By TED W. LOVE, MARSCHALL S. RUNGE, EDGAR HABER, and THOMAS QUERTERMOUS

Introduction

The concept of using antibodies to target an effector function to specific cells or molecules within the body dates from the 1950s.[1,2] Antibodies make ideal targeting vehicles because of their ability to identify selectively and adhere to an unlimited number of unique antigenic determinants. With the development of monoclonal antibody technology and

[1] D. Pressman and L. Korngold, Cancer (Philadelphia) 6, 619 (1953).
[2] L. Korngold and D. Pressman, Cancer Res. 14, 96 (1954).