Synthetic Antibodies with a Known Three-Dimensional Structure

A. Plückthun, A. Skerra, R. Glockshuber and J. Stadlmüller[1]

reprinted from:
A. Plückthun, A. Skerra, R. Glockshuber and J. Stadlmüller, Synthetic antibodies with a known three-dimensional structure. In: Protein structure and protein engineering, E.-L. Winnacker and R. Huber, Eds. (Springer Verlag, Berlin, Heidelberg, 1988), vol. 369, pp. 123-131.

The problem of discriminating self from nonself with exquisite specificity has prompted nature to develop several classes of binding proteins, of which the antibodies are the best studied. Immunoglobulins are a family of rather stable and similar molecules that are able to bind to a large number of different antigens. It is therefore tempting to carry nature's system of "protein engineering" to the laboratory.

Antibodies constitute promising targets for investigating protein-ligand interactions, since the overall folding of the domains seems to be quite independent of the structure of the binding site. Several three-dimensional structures of antibody F_{ab} fragments have been determined and their common features have been compared (Kabat 1978; Amzel and Poljak 1979; Davies and Metzger 1983). More recently, structures of complexes consisting of F_{ab} fragment and protein antigen (Amit et al. 1986; Colman et al. 1987; Sheriff et al. 1987) were solved and have brought to light again the question of conformational changes in the antigen and antibody (Marquart and Deisenhofer 1982; Huber and Bennett 1987). The common structural feature of all antibody domains is a sandwich of antiparallel β-sheets. The architecture of the variable domains is characterized by a framework of fairly constant residues (arranged in the β-sheets) linked by three hypervariable loops (complementarity determining regions, CDR) per chain. These hypervariable loops contain the residues that make contact with the antigen.

Since the framework structure is fairly well conserved between antibodies, the loop conformations as the decisive factor in determining the specificity have been the subject of theoretical studies. Several approaches to the prediction of loop conformations have been taken, including conformational search procedures combined with energy minimization or molecular dynamics (Chothia et al. 1986; Fine et al. 1986; Shenkin et al. 1987; Bruccoleri and Karplus 1987), and structural homology searches (de la Paz et al. 1986; Chothia et al. 1986; T.A. Jones and Thirup 1986). The powers and limitations of these methods have not yet clearly emerged, particularly since few comparisons between their results and experimental data have been carried out. Clearly, there is an urgent need for a sufficiently large database of experimentally determined structures. The systematic variation of single parameters (such as the length or composition of single loops) and the modification of one single antibody may provide a deeper insight into the problem. Thus, an experimental system that permits the facile alteration of the antibody sequence would be of great value.

We have approached this challenge by using as a model system the exceptionally well-characterized phosphorylcholine binding myeloma protein McPC603, an immunoglobulin A of the mouse. Previous studies of this antibody include the determination of the sequence (Rudikoff and Potter 1974; Rudikoff et al. 1981), hapten binding constants (Metzger

[1]Genzentrum der Universität München, c/o Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried bei München, FRG

39. Colloquium Mosbach 1988
Protein Structure and Protein Engineering

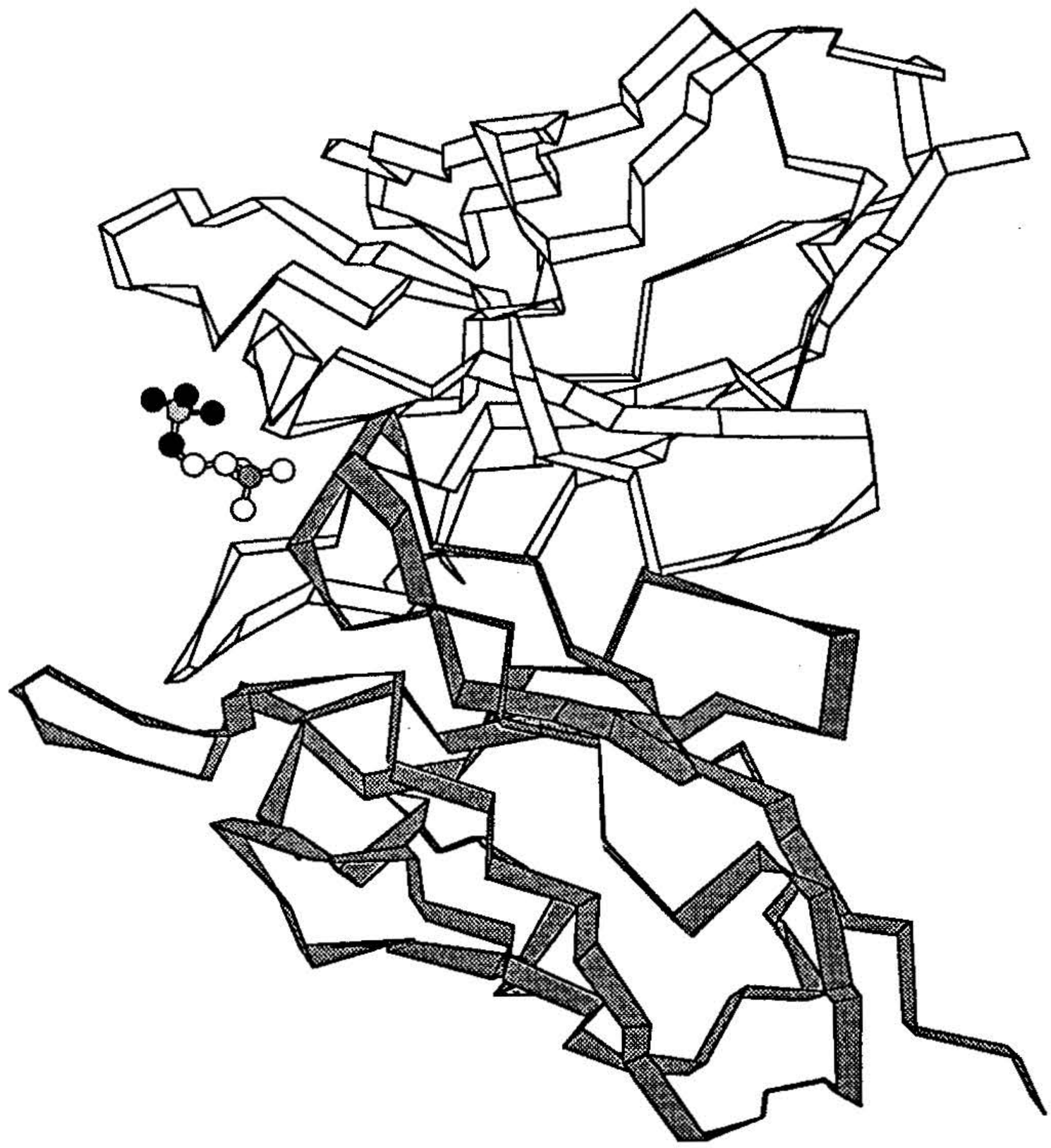

Fig. 1. Ribbon diagram of the F_V fragment of McPC603. The coordinates of Padlan et al. (entry 2MCP in the Protein Data Bank) were used with the plot programm of Lesk and Hardman (1982). The α-carbon tracing of the light chain is colored *grey* and that of the heavy chain is shown in *white*. The hapten phosphorylcholine is colored according to atom types with O in *black*, P in *light grey*, C in *white* and N in *dark grey*

et al. 1971; Leon and Young 1971; Young and Leon 1977), binding kinetics (Goetze and Richards 1977) and, most importantly, the three-dimensional structure of the F_{ab} fragment (Segal et al. 1974; Satow et al. 1986). Furthermore, sequences of several related antibodies have been determined (Potter 1977; Perlmutter et al. 1984; Kabat et al. 1987) and the interactions between the variable domains have been analyzed (Chothia et al. 1985).

The bottleneck for any protein engineering studies on antibodies was a suitable expression system. Expression of functional whole antibodies has only been reported for cells of higher eukaryotes (Morrison and Oi 1984; Morrison 1985; Roberts and Rees 1986). Yet, genetic manipulations, efficient transformations, fast growth, and simple fermentation can all be carried out more easily in bacterial systems. Expression of a native antibody or binding fragment had, however, never been reported for any bacterial system. Previous attempts have been reviewed (Morrison 1985; Boss and Wood 1985). Antibody proteins were only obtained from inclusion bodies in *E. coli* (Cabilly et al. 1984; Boss et al. 1984), but the renaturation yielded only a small amount of functional protein. Similarly, only a fraction of the antibody protein produced by yeast was functional (Wood et al. 1985). Active antibody or an antigen binding fragment had not been purified from either yeast or *E. coli*.

We solved this problem by developing an expression system (Skerra and Plückthun 1988) that was designed to reproduce in *E. coli* the assembly process of antibodies in the eukaryotic cell. There, the immunoglobulin chains are secreted into the lumen of the endoplasmic reticulum (ER), where protein folding, assembly of the light and heavy chains and disulfide-bond formation occurs. In the lumen of the ER and subsequently in the Golgi apparatus the protein is also glycosylated. Our working

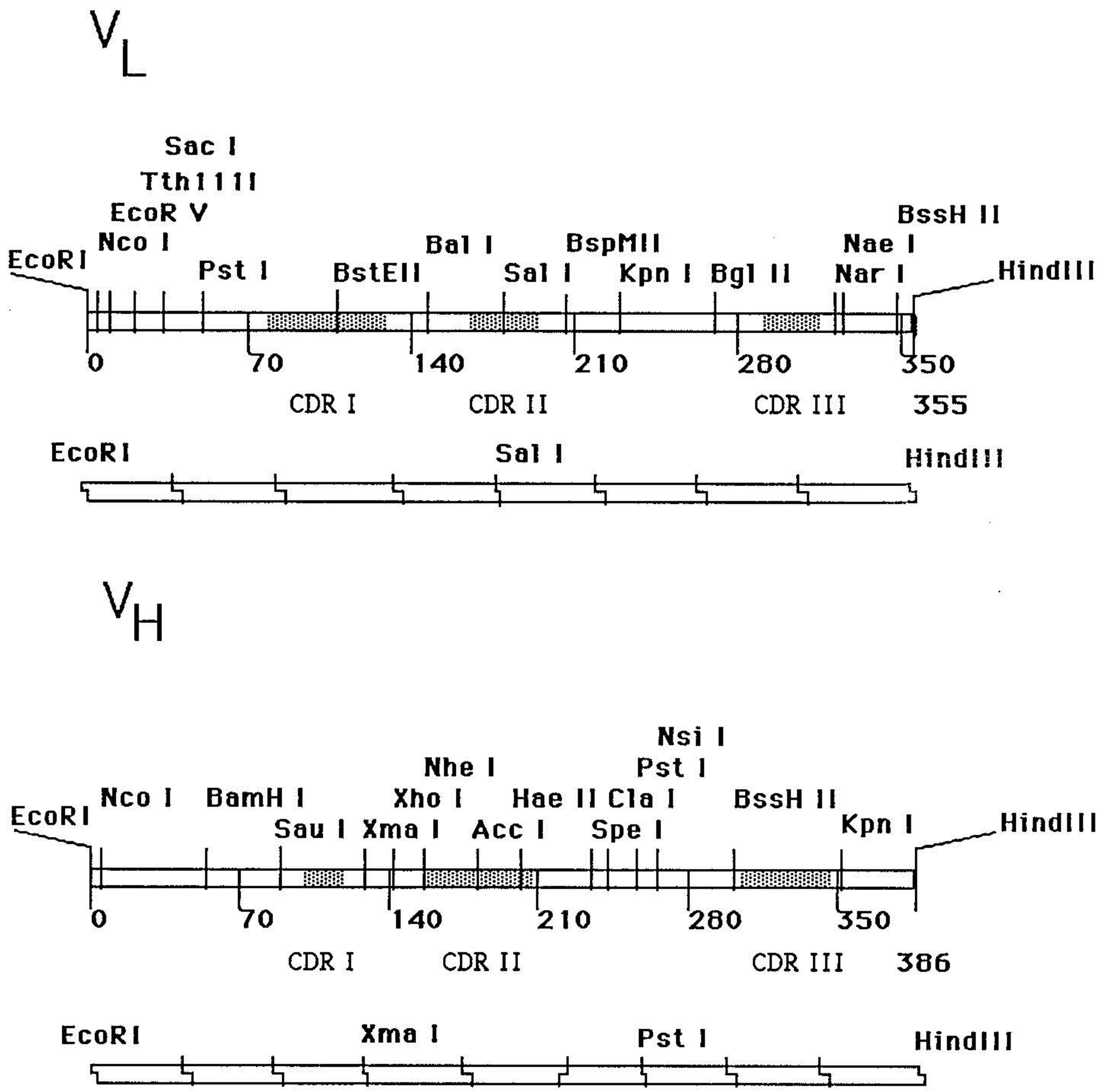

Fig. 2. Schematic representation of the synthetic genes. Unique restriction sites are indicated by a *vertical line with the label above it*. The complementarity determining regions *(CDR)* are *stippled*. The bottom representation of each gene schematically shows the oligonucleotides from which it was assembled, as well as the cloning sites used to assemble the gene from subclones (two subclones for V_L, 3 for V_H)

hypothesis was that the transport to the periplasm of *E. coli* is functionally equivalent to the transport to the ER in the eukaryotic cell. While the transport of heterologous proteins to the periplasm of *E. coli* with correct folding and correct S-S bond formation had been observed in several instances (for a review, see e.g., Briggs and Gierasch 1986) it was unknown whether two different proteins (light and heavy chain) would assemble to a heterodimer in *E. coli* in vivo. It is, for instance, not clear whether other proteins participate in the antibody assembly process in eukaryotes (Hendershot et al. 1987) and whether bacteria possess functionally equivalent proteins. A suitable bacterial expression system for functional antibody fragments must ensure (i) synthesis of approximately stoichiometric amounts of the two antibody chains, (ii) transport of both precursor proteins to the periplasmic space, (iii) correct processing of both signal sequences, resulting in the same N-termini as in the protein isolated from the mouse, (iv) folding to

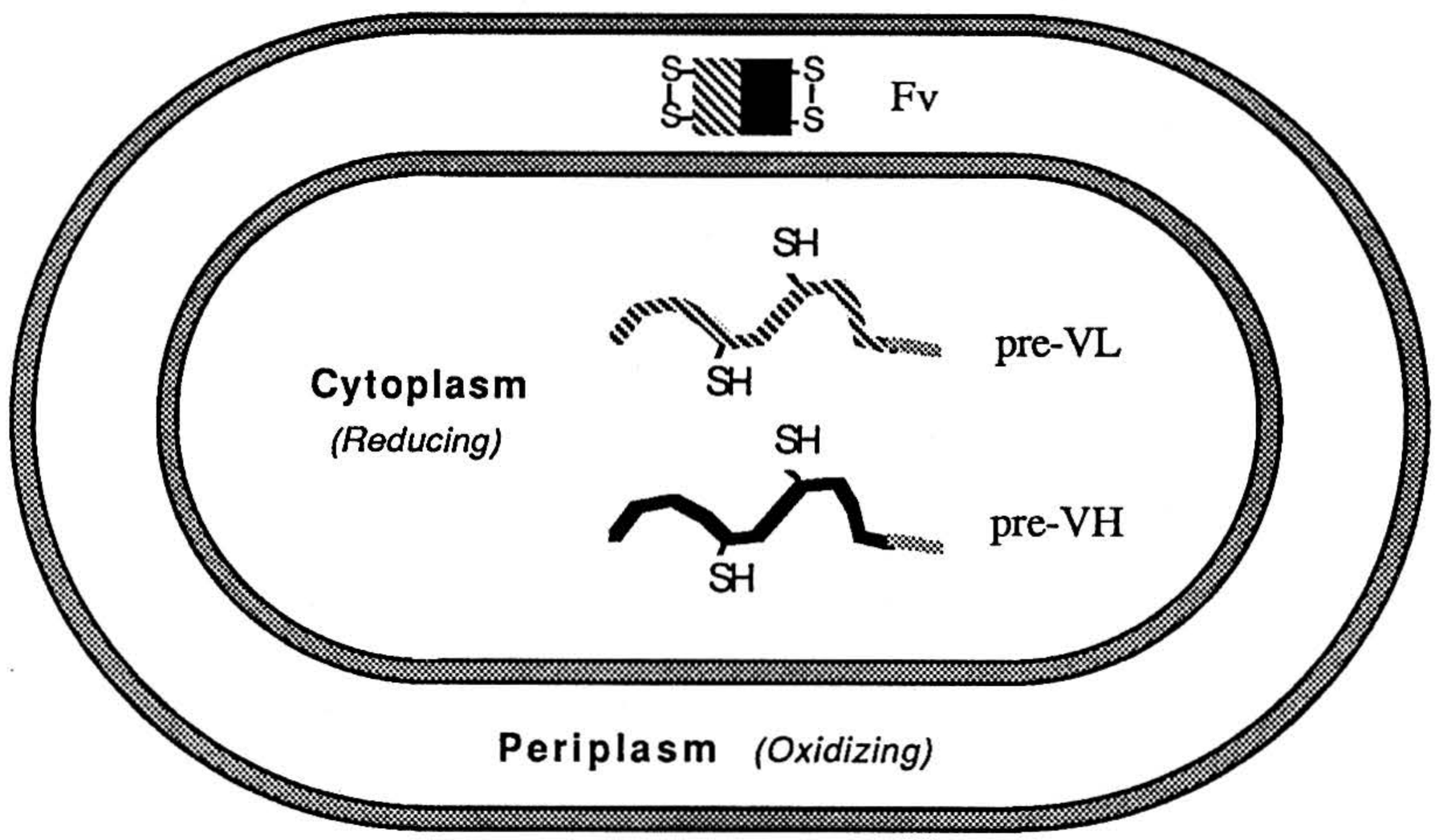

Fig. 3. Schematic diagram of the *E. coli* secretion system. In the cytoplasm, the precursor proteins for V_L and V_H, each fused to a bacterial signal sequence, are synthesized in reduced form. After translocation through the inner membrane into the periplasm, the signal sequences are cleaved off, the disulfide bonds form, and the domains fold and assemble (see text)

globular and soluble domains, (v) formation of the intramolecular disulfide bonds and finally (vi) association of the two chains to form a heterodimer.

The experiments were carried out with the F_V fragment of McPC603, which is the heterodimer of the variable domains of both the light and the heavy chain, V_L (115 amino acids) and V_H (122 amino acids) (Fig.1). Each domain contains one intramolecular disulfide bond (connecting cys 23 to cys 94 in V_L and connecting cys 22 to cys 98 in V_H). There is no disulfide bond between the chains and no other free sulfhydryl group. The genes for V_H and V_L were synthesized (Plückthun et al. 1987) (Fig.2) using the known protein sequence (Rudikoff and Potter 1974; Rudikoff et al. 1981). The gene sequences were designed to contain many unique restriction sites as well as not to contain codons very rare in *E. coli*. The two genes were fused to two different bacterial signal sequences and arranged in an artificial dicistronic operon, under the control of the inducible lac promoter. With this expression vector (Skerra and Plückthun 1988), both chains are secreted into the periplasmic space, where they assemble spontaneously to form a functional heterodimer (Fig.3). The recombinant F_V fragment so produced can be purified to homogeneity in a single step by affinity chromatography with the hapten phosphorylcholine as affinity ligand.

The functionality of the F_V fragment was tested by equilibrium dialysis with radioactive phosphorylcholine. These experiments showed that there is one binding site per F_V fragment, that there is no inactive protein in the preparation and that the affinity constant of the F_V fragment with phosphorylcholine ($1.21 \pm 0.06 \times 10^5\ M^{-1}$) is, within experimental error, identical to that of intact McPC603 isolated from mouse ascites (Skerra and Plückthun 1988). It has been debated in the literature whether F_V fragments possess the same binding affinity as the native antibodies (Inbar et al. 1972; Sen and Beychok 1986), but our experiments showed normal affinity of the hapten to the F_V fragment of MCPC60
The main conclusion from these investigations is that the F_V fragment

of McPC603 is a fully functional antibody model system for studying antigen-antibody interactions and that the very convenient *E. coli* expression system will facilitate antibody engineering.

We have also developed a second method for the expression of F_V fragments in *E. coli*. Fusion proteins between β-galactosidase and V_L or V_H were constructed in which the two proteins were linked by a recognition site for the highly specific blood clotting protease factor Xa. The denatured fusion proteins were obtained in large amounts from inclusion bodies (Plückthun et al. 1987). The F_V fragment was renatured in vitro from the combined cleavage reaction mixtures and purified by phosphorylcholine affinity chromatography (Glockshuber and Plückthun, in preparation). In the renatured F_V fragment both disulfide bonds were demonstrated to be present.

The use of bacteria as expression hosts for functional antibody fragments may open the door to new techniques that may be more difficult or impossible to achieve with other systems. The high transformation rate of *E. coli* should permit one to establish mutant libraries by using DNA that was subjected to random mutagenesis. Expression in the native state may allow the analysis of binding mutants directly on bacterial colonies. Even the use of positive metabolic selection for new functions may be envisaged, a potential very hard to realize in higher cells because of the paucity of selectable markers. In addition to the variable domains, the constant regions have been the focus of several recent investigations. For instance, marker enzymes (Neuberger et al. 1984), toxins (Möller 1982), constant regions from a different immunoglobulin class (Neuberger et al. 1985) or different species (P.T. Jones et al. 1986) are being used in experiments aimed at replacing the constant domains. The bacterial expression system may also facilitate the construction of such new hybrid antibodies.

To further understand the process of hetero-dimer assembly, we have investigated the properties of the separated domains V_L and V_H. For this separation, ion exchange chromatography in the presence of urea or reverse phase HPLC was used. The disulfide bonds remained intact during the separation procedure. It could be shown by FPLC size exclusion chromatography that V_L quantitatively dimerizes with itself (Glockshuber and Plückthun, in preparation). The sequence of the V_L domain is 63% identical to the sequence of the Bence-Jones protein REI (Palm and Hilschmann 1975), a V_L dimer from the urine of a multiple myeloma patient (Hobbs 1975). The three-dimensional structure of the REI dimer has been determined at high resolution (Epp et al. 1974,1975). The V_L domain interface is strongly conserved between a subunit of REI and V_L of McPC603. We propose, therefore, that the light chain of McPC603 dimerizes in a structure very similar to that found for REI. No indication was obtained for a dimerization of the V_H domain. A three-dimensional model for a putative V_H-dimer was generated from replacement of the V_L domain by V_H in the F_V-part of the structure of the F_{ab} fragment (Segal et al. 1974). The modeling suggested that such a structure is sterically not possible since the two CDR3 loops would collide. At high concentrations, V_H may form non-specific aggregates to aovid the exposure of the large hydrophobic inter-domain region to the solvent. It may be interesting in this context that it has been proposed that there is a specific protein in the eukaryotic cell that temporarily associates with the heavy chain (Hendershot et al. 1987).

The antibody McPC603 is also used by us as a model system for investigating enzymatic catalysis. Enzymes discriminate the transition state of the catalyzed reaction as the structure to be bound most tigthly.

By this strategy, part of the intrinsic binding energy of the ground state is converted into bringing the bound substrate closer to the transition state. The idea of transition state complementarity of enzymes was already considered by Haldane (1930) and Pauling (1946). Many transition state analogs have been synthesized and shown to be effective enzyme inhibitors (review: Wolfenden 1976). Recently, transition state analogs have also been used as haptens for immunization and were shown to indeed elicit catalytic antibodies (Raso and Stollar 1975a,b; Tramontano et al. 1986; Pollack et al. 1986; Jacobs et al. 1987).

A particularly attractive feature of McPC603 is that it binds a phosphate-ester and may therefore already be close in structure to a transition-state binding protein. It may thus stabilize the tetrahedral intermediate (and therefore, according to Hammonds principle, the transition states leading to and away from it) of, e.g., an ester hydrolysis of a properly designed substrate. The antibody may therefore act like an enzyme. Phosphates and phosphonates have been demonstrated to act as transition state analogs for enzymatic carboxyl-ester or amide hydrolysis reactions (Weaver et al. 1977; Kam et al. 1979; Hofmann and Rottenberg 1980; Jacobsen and Bartlett 1981; Thorsett et al. 1982; Bartlett and Marlowe 1983; Galardy et al. 1983; Tronrud et al. 1986; Bartlett and Marlowe 1987; Tronrud et al. 1987). Esterases and proteases have been found to be complementary in structure to the tetrahedral intermediate of the hydrolysis reaction (Kraut 1977). Recently, a serine protease that was stripped of its catalytic triad has been shown to display residual activity, presumably by transition state stabilization (Carter and Wells 1988). The antibody McPC603 is a very suitable system with which to investigate catalytic rate enhancements, since the structure of the complex with the hapten is known and since we have devised a convenient expression system that permits us to modify the protein (see above). With this system, theories on rate acceleration may therefore be directly tested.

While previous experiments with monoclonal catalytic antibodies have demonstrated the validity of Haldane's and Pauling's concepts, they have as yet provided little information on the nature of this catalysis since structural information is available for none of these antibodies. In the case of McPC603, however, the three-dimensional structure with the hapten bound is known. Protein-engineering of this molecule should deepen our understanding of catalytic requirements through experiments aimed at modifying the putative active site.

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