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## Assembly of a Functional Immunoglobulin F<sub>v</sub> Fragment in *Escherichia coli*

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## Assembly of a Functional Immunoglobulin F<sub>v</sub> Fragment in *Escherichia coli*

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An expression system was developed that allows the production of a completely functional antigen-binding fragment of an antibody in *Escherichia coli*. The variable domains of the phosphorylcholine-binding antibody McPC603 were secreted together into the periplasmic space, where protein folding as well as heterodimer association occurred correctly. Thus, the assembly pathway for the  $F_v$  fragment in *E. coli* is similar to that of a whole antibody in the eukaryotic cell. The  $F_v$  fragment of McPC603 was purified to homogeneity with an antigen-affinity column in a single step. The correct processing of both signal sequences was confirmed by amino-terminal protein sequencing. The functionality of the recombinant  $F_v$  fragment was demonstrated by equilibrium dialysis. These experiments showed that the affinity constant of the  $F_v$  fragment is identical to that of the native antibody McPC603, that there is one binding site for phosphorylcholine in the  $F_v$  fragment, and that there is no inactive protein in the preparation. This expression system should facilitate future protein engineering experiments on antibodies.

MMUNOGLOBULINS (IGS) ARE A FAMily of stable and similar molecules that can bind to a large number of different antigens. They constitute promising targets for investigating protein-ligand interactions since the overall folding of the domains seems to be independent of the structure of the binding site. Several three-dimensional structures of antibodies or their Fab fragments have been determined, and their common features have been compared (1). The essence of their architecture is a framework of fairly constant residues (arranged in a sandwich of  $\beta$ -sheets) linked by three hypervariable loops [complementarity-determin-

ing regions (CDR)] per chain that determine the specificity for antigen recognition. An early insight into enzyme catalysis by Pauling (2) was used to search for catalytic antibodies (3). An easy access to genetically engineered, functional antibody proteins would permit new approaches for studying antibody structure and function and the essentials of enzymatic catalysis (4). Despite numerous investigations, the

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expression of functional whole antibodies or functional antigen-binding fragments of antibodies has not been reported for any bacterial expression system, and the chance of designing such an expression system has been viewed pessimistically (5, 6). The expression of antibodies in yeast has been described (7), but only a small fraction of the expressed protein was functional. The purification of active antibodies or antibody fragments from yeast or any other microorganism has not been reported. In E. coli, the antibody protein could be produced only in a non-native state (8), and refolding experiments led to only a small percentage of correctly folded recombinant antibodies. Moreover, it is difficult to purify the native

the production of functional antibodies (5, 9, 10). Yet none of these expression systems compares to E. coli in the ease of genetic manipulation, efficient transformation, fast growth, simple fermentation, and favorable economics. A bacterial expression system in which the chains assemble to form a functional complex in the same cell would permit the use of assays directly on bacterial colonies. One could then use positive selection methods for antigen binding and possible catalytic functions of the mutant antibodies. Apart from the investigation of the variable regions themselves, the possible replacement of the constant regions through genetic means by marker enzymes (11), toxins (12), or Ig regions from a different class (13) or from a different species (14) has attracted attention. The production of such hybrid antibodies may also be facilitated by a bacterial expression system.

decided on an antibody with a known amino acid sequence and a three-dimensional structure that may also be close to a transitionstate binding protein. Such a system is the myeloma protein McPC603 (15, 16), a phosphorylcholine-binding IgA from mouse. We decided to investigate the expression of the F<sub>v</sub> fragment of McPC603 in E. coli. This fragment is the dimer of the  $V_L$  (115 amino acids) and  $V_H$  (122 amino acids) domains and contains the whole antigen binding site. Each domain has one intramolecular disulfide bond (connecting Cys-23 to Cys-94 in V<sub>L</sub> and connecting Cys-22 to Cys-98 in  $V_H$ ). There is no disulfide bond between the chains and no other free cysteine. We synthesized the genes for both the  $V_L$  and the  $V_H$  domain. The exact DNA sequence we synthesized, the synthesis methodology, and the logic of the sequence design are discussed elsewhere (4).The expression system described herein is the result of attempts to reproduce in E. coli the folding and assembly pathway of antibodies in eukaryotic cells. In the eukaryotic plasma cell, the two chains of an antibody are separately transported from the cytoplasm to the lumen of the endoplasmic reticulum (ER) (17). This transport requires an NH<sub>2</sub>-terminal signal sequence, which is cleaved off during or after the translocation event by a signal peptidase, to produce the mature protein. In the lumen of the ER, protein folding, formation of the disulfide bonds, and the association of individual chains to form the functional antibody take place (17). It is not clear yet which other proteins play an essential role in mediating these folding and assembly processes. In addition to these critical steps, which must be mimicked in the bacterial cell in order to obtain a functional F<sub>v</sub> fragment, the antibodies are glycosylated in the lumen of the ER and Golgi apparatus and transported to the cell surface. Usually only the F<sub>c</sub> is glycosylated, but this glycosylation is not required for antigen binding. Our hypothesis was that the protein transport to the periplasm of E. coli is functionally equivalent to the transport of a protein to the lumen of the ER of a eukaryotic cell. We developed a system for expressing both chains in the same E. coli cell and secreting them together into the periplasm of E. coli. This system should permit the following critical steps in the assembly of a functional F<sub>v</sub> fragment to occur: (i) synthesis of approximately stoichiometric amounts of both chains, (ii) transport of both precursor proteins to the periplasmic space, (iii) correct processing of both signal sequences, resulting in the same NH2-termini as in the protein isolated from the mouse, (iv) fold-

protein from non-native contaminants, which complicates accurate measurements of binding constants, folding yields, and spectral properties.

Other investigators preferred expression in cells of higher eukaryotes, thus permitting

Fig. 1. Plasmid pASK22 for the coexpression and cosecretion of the  $V_L$  and  $V_H$  domain of McPC603 in E. coli. The plasmid contains the origin of replication (ori) and the ampicillin resistance gene (Ap<sup>r</sup>) from the pUC family of plasmids (29). The V<sub>H</sub> and the V<sub>L</sub> domain are encoded as two separate proteins on the same transcription unit downstream from a lac promoter-operator ( $lac^{p/o}$ ) (29), which is inducible by isopropyl- $\beta$ -D-thiogalactoside (IPTG). The synthetic genes encoding the  $V_H$  and the  $V_L$  domain (4) are precisely fused to gene fragments encoding the signal sequence of the outer membrane protein A (ompA) (19) and the alkaline phosphatase (phoA) (20), respectively. Each coding region is preceded by a ribosomal binding site to ensure efficient translation initiation. Thus, both genes are arranged in a regulatable artificial dicistronic operon. The construction of the plasmid was performed by using standard DNA methodology (30).

In our search for an antibody model for investigations on binding and catalysis, we



Fig. 2. Purification of the  $F_v$  fragment. An SDS-PAGE (14%) (31) stained with Coomassie brilliant blue is shown. (Lane 1) Total cell protein; (lane 2) periplasmic fraction; (lane 3) purified F<sub>v</sub> fragment; and (lane 4) protein molecular size marker. For the purification, a culture of E. coli strain W3110 harboring plasmid pASK22 was grown in lactose broth (LB) medium containing ampicillin (100 mg/liter) to attain an absorbance at 550 nm of 0.5. After induction for 45 min by addition of isopropylthiogalactoside to a final concentration of 1 mM, the cells were harvested by centrifugation at 4000g for 10 min (at 4°C). A cell fractionation was carried out by resuspending the cell pellets in TES buffer (0.2M tris-HCl, pH 8.0; 0.5 mM EDTA; 0.5M sucrose) (10 ml per liter of original culture). The cells were then subjected to a mild osmotic shock by addition of TES, diluted 1:4 with H<sub>2</sub>O, and containing 2 mM phosphorylcholine (15 ml per liter of original culture). After incubation on ice for 30 min, the suspension was centrifuged (5000g, 10 min) and the supernatant was centrifuged again (48,000g, 15 min). This supernatant, which contained all soluble periplasmic proteins (32), was concentrated by ultrafiltration with an Amicon YM 5 membrane to a volume of approximately 2.5 ml per liter of original culture and dialyzed against BBS buffer (0.2M borate/NaOH pH 8.0, 0.16M NaCl). This concentrated solution was applied to a phosphorylcholine affinity column (2.5-ml bed volume per 4 liters of bacterial culture) (33). After washing with BBS, pure Fv fragment was eluted with a solution of 1 mM phosphorylcholine in BBS. The typical yield of this not yet optimized procedure is approximately 0.2 mg of purified Fv fragment per liter of bacterial culture.



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ing to globular and soluble domains, (v) formation of the intramolecular disulfide bonds, and (vi) association of the two chains to form a heterodimer. Several examples (18) illustrate the secretion of heterologous monomeric proteins into the periplasm of *E. coli*, but it was not known whether folding and assembly of two different subunits can also occur to form a functional dimeric protein.

The expression vector we constructed is schematically drawn in Fig. 1. The genes, precisely fused to bacterial signal sequences (19, 20), are arranged in an artificial dicistronic operon. A homogeneous  $F_v$  fragment can be prepared from the periplasmic fraction of a cell lysate in a single step by affinity

indicate that folding and hetero-association of the variable domains is possible without known external help and is strongly favored in the periplasm of E. coli. Thus we could show that even when the assembly of two different chains is necessary for the formation of a functional protein, the transport to the periplasm of E. coli is functionally equivalent to the eukaryotic transport to the lumen of the ER. Further experimentation will be needed to clarify whether there is any role of homodimers (Bence-Jones proteins) (25) as assembly intermediates, or whether the correct heterodimer association is both kinetically and thermodynamically favored over homodimer formation. Most of the soluble V<sub>L</sub> and V<sub>H</sub> protein from the periplasmic fraction binds to the affinity column, indicating that it is correctly assembled to a heterodimer.

antibody McPC603. This finding could not be expected a priori, since there is considerable debate about the functionality of  $F_v$ fragments (26, 27). The first accurate study of an F<sub>v</sub> fragment focused on the dinitrophenol (DNP)-binding antibody MOPC-315. It revealed that the affinity constants for DNP were essentially identical for the F<sub>v</sub> fragment and the Fab fragment (26). In a recent investigation of the human riboflavin-binding antibody Gar (27), a fragment consisting of  $V_H$  and the whole light chain was prepared. This fragment, which is comparable to an F<sub>v</sub> fragment, has an affinity constant for riboflavin that is about three orders of magnitude lower than that determined for the native antibody. These results were contradictory, and it was not clear whether they are the consequence of true differences between antibodies (28) or are the result of experimental side effects (27). We conclude that the Fv fragment of McPC603 is fully functional and can serve as a convenient model for studying antigenantibody interactions, since the three-dimensional structure of the corresponding Fab fragment is known (16). We have devised an expression system not requiring any in vitro manipulations such as cleavage of fusion proteins, oxidation, or refolding. Furthermore, expression in a functional state permits the use of hapten binding for rapid and selective purification. The periplasmic location of the protein reduces both the potential protease degradation problem and the number of contaminating protein species to be separated. We believe that protein engineering of antibodies is greatly facilitated with this expression system.

chromatography (legend to Fig. 2).

As can be deduced from the SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2), the F<sub>v</sub> fragment is completely pure. Both chains of the purified F<sub>v</sub> fragment are present in a 1:1 molar ratio and have the sizes expected for the mature proteins (V<sub>H</sub>, 13,600; V<sub>L</sub>, 12,400). To confirm the correct cleavage of both signal sequences, the six amino-terminal amino acids of the two chains [V<sub>H</sub>, NH<sub>2</sub>-Glu-Val-Lys-Leu-Val-Glu; V<sub>L</sub>, NH<sub>2</sub>-Asp-Ile-Val-Met-Thr-Gln; (15)] were sequenced (21, 22). Both heterologous fusion proteins were properly cleaved by the bacterial signal peptidase, and there was no indication of either imprecise processing or any NH2-terminal degradation.

We measured the affinity constant of the recombinant F<sub>v</sub> fragment for phosphorylcholine by equilibrium dialysis (Fig. 3). The same conditions were used as in the determination of the affinity constant of native McPC603 isolated from mouse ascites (23). The value found for the F<sub>v</sub> fragment  $(1.21 \pm 0.06 \times 10^{5} M^{-1})$  (Fig. 3) is identical (within experimental error) to that reported (23) for the native antibody  $(1.6 \pm 0.4 \times 10^5 M^{-1})$ . The Scatchard plot (Fig. 3) is linear and extrapolates to approximately 1 mole of hapten bound per mole of F<sub>v</sub> fragment. This shows that there is one binding site per F<sub>v</sub> fragment and that there is no inactive protein in the preparation. We conclude that it is possible to express the  $F_v$  fragment of McPC603 as a fully functional and stable protein in E. coli. There was no previous indication of whether E. coli would be able to assemble a protein consisting of different subunits in the periplasm. Escherichia coli seems to assemble its own proteins by a different method. Escherichia coli penicillin-acylase, the best characterized protein that fits the definition of a soluble heterodimeric protein in the periplasm, is proteolytically processed from a single chain precursor in the periplasm (24). Our results Our second result is that the  $F_v$  fragment of McPC603 has essentially the same affinity constant for phosphorylcholine as the intact



Fig. 3. Equilibrium dialysis data for the binding of phosphoryl[methyl-14C]choline to the recombinant F<sub>v</sub> fragment of McPC603. The equilibrium dialysis was carried out in a multicavity microdialysis chamber (Bel-Art Products) with a volume of about 100 µl on each side of the membrane. The chambers were filled with 50  $\mu$ l of purified F<sub>v</sub> fragment in BBS on one side and 50 µl of a solution of phosphoryl[methyl-14C]choline (50 mCi/mmol, Amersham) in BBS on the other side. The concentration of the F<sub>v</sub> fragment was determined to be 0.22 mg/ml from an  $A_{205}$  of 6.85 [using an extinction coefficient of  $\epsilon_{1cm}^{1mg/ml} = 31$ for the average absorption of the peptide bond region in proteins at 205 nm (34)]. After equilibration for 22 hours at ambient temperature, samples (20 µl) from each solution were counted in 5 ml of Rotiszint 22 (Roth Biochemicals) using a Beckman LS 1801 scintillation counter. The data obtained are plotted according to Scatchard (35). An r denotes the fraction of antibody fragment with bound hapten, and c denotes the concentration of free hapten. The line was fitted by linear regression analysis. From the slope of line this affinity an constant of  $K_{\rm a} = 1.21(\pm 0.06) \times 10^5 M^{-1}$  is obtained.

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32. We monitored the selectivity of the cell fractionation

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