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ENGINEERING AND HETEROLOGOUS EXPRESSION OF ANTIBODIES IN *ESCHERICHIA COLI*

PLUCKTHUN, A.

Genzentrum der Universität München, c/o Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

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Antibodies are widely used in biochemical research, medical diagnostics and therapy. They are also of high interest to basic research in protein engineering as they display selective recognition of an enormous variety of molecules, yet are all built with the same chain fold. Both areas, applied and basic research, benefit from being able to engineer antibodies.

While the recombinant DNA techniques necessary for protein engineering have been well established for some time, the problem with antibodies had been the expression of the modified protein. In the past, functional expression of antibodies was limited to cells of higher eukaryotes (for recent reviews, see e.g. 1,2). However, the production of antibodies in bacteria would facilitate not only protein engineering by the convenient handling and facile fermentation that bacteria allow, but also permit for the first time to make use of the powerful techniques of bacterial genetics: Since the transformation and transfection of bacteria is very efficient, libraries can be established (see below), and random mutagenesis can be carried out with bacterial plasmids. Since much larger number of bacterial cells can be handled than eukaryotic cells, screening of mutants and libraries becomes possible with bacteria. Even the use of metabolic selections is conceivable with bacteria. It should be noted that all screening approaches require the expression of the antibody or of an antigen binding fragment in the native state.

We recently developed such an expression system, with which the expression in the native state can be achieved in *Escherichia coli* (3,4). This expression system was designed to achieve co-expression and co-secretion of both chains to the periplasm of *E. coli* (either only the variable domains V_L and V_H to give the F_v fragment, or the complete light chain (V_LC_L) and the first two domains of the heavy chain (V_HC_H) to give the F_{ab} fragment). We showed that both chains fold, their disulfide-bonds form and they associate to the correct heterodimer in the periplasm of *E. coli*. This expression in the native state allows the recombinant F_v or F_{ab} fragment to be purified from *E. coli* by a hapten-affinity chromatography in a single step. This expression strategy has now been extended to antibody libraries (5).

Our experiments were carried out with the especially well characterized antibody McPC603 as a model system. McPC603 is a phosphorylcholine binding IgA of the mouse. This protein was originally obtained as a myeloma protein (6), and had been extensively characterized. The sequence, the crystal structure of its F_{ab} fragment (7,8), as well as binding constants and binding kinetics of several haptens had been determined (see e.g. 9; reviewed in 10), facilitating the characterization of recombinant products. We obtained the genes for the variable domains synthetically (encoding the F_v fragment) and also linked them to appropriate, cloned constant domains (to encode the F_{ab} fragment) (10).

F_v and F_{ab} fragments produced in *E. coli*

Amino acid analyses after derivatization of any free cysteine with 4-vinyl-pyridine showed that both disulfide bonds of the recombinant F_v fragment were quantitatively formed (Glockshuber and Plückthun, in preparation). To investigate whether both intramolecular disulfide bonds are required for the correct folding of the variable domains *in vitro*, the native fragment was reduced. The reduction was accompanied by denaturation and precipitation, and renaturation was only possible under oxidizing

conditions (Glockshuber and Plückthun, in preparation). Additionally, the problem was investigated *in vivo* by genetic engineering. In one experiment, both cysL23 and cysL94 were changed to alanine residues and in another experiment, both cysH22 and cysH98 were changed to alanine residues. From neither double mutant, any functional protein could be isolated, indicating that the presence of both disulfide bonds is essential for the correct folding of the F_v fragment both *in vivo* and *in vitro*. Both equilibrium dialysis (3) and fluorescence measurements showed (11) that there is no significant difference in the intrinsic association constant of the hapten to the whole antibody from mouse and the recombinant F_v fragment from *E. coli*.

The F_{ab} fragment was obtained and purified from *E. coli* with a strategy analogous to that used for the F_v fragment (4). Its binding constant is also indistinguishable from the native antibody or the proteolytic F_{ab} fragment although the antibody is glycosylated in the C_H1 domain (Skerra, Glockshuber and Plückthun, in preparation). Interestingly, the fraction of correctly folded protein is consistently smaller than that of the F_v fragment under identical conditions (Skerra and Plückthun, in preparation). This experimental system has been used by us as a model for studying several factors that may influence the *in vivo* folding of a heterologous, dimeric protein. To scrutinize the influence of disulfide isomerization in determining the folding yield, the second intra-domain disulfide bond connecting cysH198 and cysH222 within the C_H1 domain in mouse IgA was removed. A disulfide-rearrangement might constitute a slow folding step and thus lead to the accumulation and aggregation of a folding intermediate. However, neither the presence of this disulfide bond nor any other cys→ala substitution in the constant domains influenced the folding yield beyond experimental error. Additionally, the replacement of a *cis*-proline containig loop by a loop most likely containing a *trans*-proline residue, led to only a small effect on folding yield. It is possible that efficient folding *in vivo* requires the presence of additional factors in the cell.

The stability of the F_V fragment

To investigate the properties of the individual domains, the variable domains V_L and V_H were separated by anion-exchange chromatography in the presence of urea under non-reducing conditions. Both chains were then renatured by the removal of the denaturant. Experiments with FPLC size exclusion chromatography showed that, at least under the conditions of the chromatography experiment, V_L dimerized quantitatively (12, Glockshuber and Plückthun, in preparation). The crystal structure of this V_L-dimer has now been determined to 2.0 Å resolution (12) and refined to an R-factor of 14% (Steipe, Huber and Plückthun, in preparation). In contrast, no ordered homo-dimerization of V_H could be demonstrated. Chromatography results indicate that V_H exists as monomers and dimeric aggregates with mixed and not defined molecular shapes.

Crosslinking experiments with F_V at different dilutions in the presence and absence of the hapten phosphorylcholine have shown that there is a concentration-dependent equilibrium for the association of V_L with V_H, which is shifted towards the F_V fragment in the presence of phosphorylcholine (11). The system can be described as follows:

$$F_V + PC \overset{K_{PC}}{\rightleftharpoons} F_V \cdot PC$$

$$V_H + V_L \overset{K_{HL}}{\rightleftharpoons} F_V$$

$$V_L + V_L \overset{K_{LL}}{\rightleftharpoons} (V_L)_2$$

While the hapten-binding equilibrium constant K_{PC} is accurately known both from fluorescence and from equilibrium dialysis to be $1.6 \times 10^5\ M^{-1}$, the other two equilibria had to be estimated from crosslinking and fluorescence dilution experiments (11). K_{HL} is probably about $10^6\ M^{-1}$, and K_{LL} somewhat smaller and not yet accurately determined.

A systematic mutagenesis study and binding analysis with a variety of synthetic haptens and analogs has been carried out and will provide a useful database, against which theoretical methods for the prediction of binding constants can be tested and calibrated (Glockshuber, Stadlmüller and Plückthun, in preparation). We found for example that the positive charge of the quaternary ammonium group must be counterbalanced by exactly one negatively charged residue at the bottom of the binding pocket. No other combination of residues tested led to binding. The binding of the phosphate moiety, on the other hand, depends on the interactions with an arginine residue and a tyrosine residue. The only other combination of residues leading to any binding at all was the substitution of Tyr by Phe. This shows that there are very stringent requirements for the functionality of the binding pocket. The continuation of this study will delineate more clearly the different contributions to the observed free energy of binding and help us understand and eventually design optimal binding sites or optimal ligands.

Catalytic activity of the F_V fragment of McPC603 expressed in *E. coli*

The active sites of many enzymes have been found to be complementary in structure to the transition state of the reaction they catalyze. Part of the intrinsic binding energy of the ground state can be used in such enzymes to bring the bound substrate closer to the transition state and facilitate the reaction. Indeed, many compounds resembling the transition state ("transition state analogs") have been synthesized and been found to be excellent inhibitors, binding to the enzymes with higher affinity than the substrate. Jencks was the first to suggest eliciting antibodies against transition state analogs as potential catalysts, and several investigators have since used this strategy successfully, although the rate accelerations have not been overwhelming (for reviews, see e.g. 14,15).

In contrast, our strategy has been to develop methodologies to modify the catalytic protein itself (10,16). The protein engineering approach also allows the introduction of potential nucleophiles and acid/base catalysts into the binding site of the protein, since transition state stabilization is only one of many mechanistic devices used in enzyme catalysis. Protein engineering is needed, therefore, to combine the various catalytic factors into the same protein if catalytic efficiencies approaching those of enzymes are ever to be achieved.

The antibody McPC603 is a particularly suitable model system for investigating the structural requirements for catalysis since the three-dimensional structure is known, and we have developed a convenient expression system for producing modified protein. McPC603 binds phosphorylcholine, a phosphate ester, and should therefore be capable of binding the tetrahedral intermediate of the hydrolysis of an analogous carboxylate ester or a carbonate ester better than the planar ester substrate itself. The recombinant F_V fragment of McPC603 obtained from *E. coli* was tested for the catalysis of the hydrolysis of choline-*p*-nitrophenyl carbonate. Indeed, a rate acceleration with a K_M value of about 1.3 mM and a k_{cat} value of about 0.05 min^{-1} could be demonstrated (16). The systematic modification of both the substrate and the antibody in combination with X-ray structure analysis is now being carried out, and further insight into the exact requirements for efficient catalysis should be obtainable.

The immunization with appropriately designed transition state analogs will certainly provide a good starting point for a catalytic antibody, but a combination of this approach with the modification of a moderately active catalytic antibody by either "rational engineering" or random mutagenesis will probably be required for catalytic antibodies to become useful reagents in research, technology and medicine. The *E. coli* expression system described here may be particularly useful in this respect.

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