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Engineering of F_v and F_{ab} Fragments of the Antibody McPC603 Expressed in *E. coli*

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While techniques for altering protein sequences by gene technology are firmly established, the difficulties in the expression of antibody molecules have long been an obstacle for antibody engineering. Some time ago, we developed an *E. coli* expression system that combines the advantages of expression in the native state with those of *E. coli* as expression host[1]. *E. coli* as an expression host has a number of attractive features : Genetic manipulations are simple, fermentation is relatively straightforward, and transformation is efficient. The *E. coli* expression system may also be suitable for the random mutagenesis of antibodies.

As a model system, we used the especially well characterized antibody McPC603, a phosphorylcholine binding IgA of the mouse. The sequence, the crystal structure of its F_{ab} fragment, as well as binding constants and binding kinetics of several haptens had been determined (reviewed in ref. 2), 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)[3].

The expression system is 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)[1,4]. Both chains fold, their disulfide-bonds form and they associate to the correct heterodimer. This expression in the native state allows the recombinant F_v or F_{ab} fragment to be purified from *E. coli* with extraordinary ease: by the use of a hapten-affinity column in a single step.

Stability and folding *in vivo*: Amino acid analyses after reaction with 4-vinyl-pyridine showed that both disulfide bonds of the recombinant F_v fragment were quantitatively formed. To investigate whether both intramolecular disulfide bonds are required for the folding of the variable domains *in vivo*, two mutant F_v fragments were constructed. In one case, cysL23 and cysL94 were both changed to alanine residues and in the other case, cysH22 and cysH98 were both changed to alanine residues. In both double mutants, no functional protein could be isolated from *E. coli*, indicating that the presence of both disulfide bonds is absolutely essential for folding of the F_v fragment *in vivo*

The F_{ab} fragment was obtained and purified from *E. coli* with a strategy analogous to that used for the F_v fragment[4]. Interestingly, the fraction of correctly folded protein is consistently smaller than for the F_v fragment under identical experimental conditions. This 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 investigate the influence of disulfide isomerization in determining the folding yield, the second intradomain disulfide bond within the C_H1 domain in mouse IgA connecting cysH198 and cysH222 was removed. A disulfide-rearrangement might constitute a slow folding step and thus lead to the accumulation and aggregation of a folding intermediate. However, this disulfide bond in C_H1 was not found to influence the folding yield beyond experimental error since a double mutant carrying alanine residues in both positions yielded a similar amount of recombinant F_{ab} fragment as the wild-type with the disulfide bond present.

Hapten affinity: Both equilibrium dialysis[1] and fluorescence measurements showed that there is no significant difference in the intrinsic association constant of the hapten to the whole antibody from mouse, the F_{ab}' fragment prepared by proteolysis of the mouse antibody, the recombinant F_{ab} fragment from *E. coli* or the recombinant F_v fragment from *E. coli*.

In the F_v fragment, the two domains V_H and V_L, which are only non-covalently associated, dissociate at high dilution, leading to a dependence of the measured (apparent) hapten binding constant on protein concentration. If these two domains making up the F_v fragment are covalently crosslinked, on the other hand, a value identical to all other hapten association constants is found[6]. This indicates that the recombinant F_v fragment has the same intrinsic binding constant as all other fragments. The F_v fragment as well as the recombinant F_{ab} fragment are therefore suitable model systems for the study of antigen-antibody interactions.

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. Such a study will more clearly delineate different contributions to the

observed free energy of binding and aid in our understanding of the design of optimal binding sites and optimal ligands.

Association of the variable 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, under the conditions of the experiment, V_L dimerized quantitatively[2,5]. The crystal structure of this V_L-dimer has now been determined[7].

On the other hand, no ordered dimerization of V_H with itself could be demonstrated. This indicates that V_H exists as monomers and dimeric aggregates with a mixture of molecular shapes and not a defined dimer conformation.

Crosslinking experiments 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. 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} could be measured very accurately both by fluorescence and equilibrium dialysis techniques and was found to be 1.6×10^5 M^{-1}, the other two equilibria have to be estimated from crosslinking and fluorescence dilution experiments. K_{HL} is probably about 10^6 M^{-1}, and K_{LL} is probably of the same order of magnitude

The antibody as a catalyst: 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 may be used to bring the bound substrate closer to the transition state. 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 (reviewed e.g. in ref. 5)

In contrast, our strategy has been to develop methodologies to modify the catalytic protein itself[3]. This approach also allows the introduction of potential nucleophiles and acid/base catalysts into the protein. The antibody McPC603 is a 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 analogous carboxylate esters or of carbonate esters 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 1.5 mM and a k_{cat} value of about 0.1 min^{-1} could be demonstrated. 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.

While immunization with appropriately designed transition state analogs will certainly provide a good starting point for a catalytic antibody, a combination of both approaches, i. e. 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 for this strategy.

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