Catalytic antibodies: contributions from engineering and expression in *Escherichia coli*

reprinted from:
A. Plückthun and J. Stadlmüller, Catalytic antibodies: Contributions from engineering and expression in Escherichia coli. Ciba Foundation Symposium 159, 103-112; discussion 112-117 (1991).

Andreas Plückthun and Jörg Stadlmüller

Genzentrum der Universität München, c/o Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried, Federal Republic of Germany

Abstract. Antibodies have been raised against the transition state of many reactions and shown to catalyse the relevant reaction. Their moderate catalytic efficiencies can be increased by protein engineering, if ways can be found to express the engineered antibody. We have developed a system by which fully functional Fv and Fab fragments can be expressed in *Escherichia coli*. The Fv fragment dissociates at low concentrations; we therefore devised methods to stabilize the fragment. We showed that the Fv fragment of the antibody McPC603, a phosphorylcholine-binding immunoglobulin A, binds the antigen with the same affinity as does the intact antibody isolated from mouse ascites. Phosphorylcholine is an analogue of the transition state for the hydrolysis of choline carboxylate ester. The Fv fragment of McPC603 catalysed this hydrolysis. Mutational analysis of the residues in the binding site of the antibody has shown which are essential for binding and for catalysis, and the importance of charged residues in certain positions. The *E. coli* expression system combined with protein engineering and screening methods will facilitate understanding of enzyme catalysis and the development of new catalytic antibodies.

1991 Catalytic antibodies. Wiley, Chichester (Ciba Foundation Symposium 159) p 103–117

Enzymes use a large repertoire of mechanistic devices to accelerate chemical reactions. One is to exploit the structure of the active site itself. Its shape, hydrophobicity, hydrogen bonds and electrostatic potential not only ensure specific binding of only one or a few substrates, but also contribute in many enzymes to the acceleration of the reaction (for review see Kraut 1988). In enzymes that use catalysis by transition state stabilization, the structure of the active site is more complementary to the transition state of the reaction than to the ground state (Haldane 1930, Pauling 1946). This hypothesis predicts that stable inhibitors which resemble the fleeting transition state should bind more

tightly to the enzyme than does the substrate. This prediction has been verified by many experiments (Wolfenden 1976).

Other mechanisms used by enzymes to speed up a reaction include general acid-base catalysis, Lewis acid catalysis, entropic effects, covalent catalysis and the use of coenzymes (Jencks 1969, Walsh 1979, Fersht 1984). Metal ions and coenzymes are the means by which the chemistry of the 20 natural amino acids can be extended. Obviously, the type of reaction to be catalysed determines the mechanisms by which it can be accelerated.

Antibodies share with enzymes the ability to bind chemical compounds rather selectively; this is true for a wide spectrum of different molecules. Moreover, antibodies can be raised specifically to defined compounds. The decisive idea in using antibodies for catalysis was to raise them not to the ground state, but to analogues of the transition state of the reaction, thereby diverting part of the intrinsic binding energy to bringing the substrate closer to the transition state and thus accelerating the reaction (Jencks 1969). The rate acceleration achievable by this strategy is not dramatic, but a whole spectrum of different reactions has now been catalysed (reviewed in e.g. Powell & Hansen 1989, Schultz 1989, Shokat & Schultz 1990). Pioneering investigations (Slobin 1966, Raso & Stollar 1975a,b, Kohen et al 1979, 1980) were hampered by the problem that in polyclonal serum high enough concentrations of the catalytic species are not available, because the antibodies elicited in response to the immunization of an animal make up only a fraction of the total immunoglobulin in the serum. The discovery of monoclonal antibodies (Köhler & Milstein 1975) has made the detailed investigation of antibody-mediated catalysis possible.

The rate accelerations achievable with monoclonal antibodies raised against transition state analogues are comparable to those observed with an enzyme that has been stripped of chemical catalysis. Such an experiment has been performed with subtilisin (Carter & Wells 1988, 1990). The whole catalytic triad (Ser-221, His-64, Asp-32) was replaced by alanine residues. As expected, the rate acceleration was greatly reduced; nevertheless, a k_{cat} of $3 \times 10^{-5}\,s^{-1}$ was observed, which is 2700-fold faster than the rate in solution. This residual catalysis is probably caused by the structural complementarity of the enzyme to the transition state.

Clearly, for high rate accelerations using antibodies, several mechanistic devices have to be combined, just as in 'real' enzymes. Although the immunological repertoire is enormous, it remains doubtful whether immunogens can be devised (except maybe in special cases) that elicit natural antibodies containing, for example, nucleophilic groups or metal-binding sites. The basis of antibody development is selection for tight binding, but this is not necessarily the major determinant of efficient catalysis. Acid-base catalysts, for instance, function optimally if their pK_a is about equal to the pH of the reaction mixture. The immune system, however, selects for *strong* acids and *strong* bases to complement charges in the antigen. Metal-binding sites can probably be

created by a purely immunological approach only if a chelating agent is part of the immunogen (Iverson & Lerner 1989), and a metal chelate must then be used as a cofactor. Furthermore, problems will be encountered in screening, if the antibody-producing cells themselves make enzymes with specificities similar to those of the antibody-catalysed reactions for which one is screening.

Why *Escherichia coli*?

These considerations made it desirable to develop methods of protein engineering by which to modify the antibody molecule itself. The techniques for modification of DNA sequences were well established, but no convenient methods were available for expressing the engineered antibody protein. A large amount of work has been done on the expression of antibodies in eukaryotic cells (for reviews, see Brüggemann & Neuberger 1988, Morrison & Oi 1989), but these do not offer the same flexibility or convenience that an *E. coli* expression system does.

Unfortunately, previous attempts to express antibodies and antibody fragments in *E. coli* (Boss et al 1984, Cabilly et al 1984) produced very little functional protein. Most of the protein was obtained as insoluble inclusion bodies. It is, however, the expression in fully functional form that would make the *E. coli* system particularly interesting for antibodies and antibody fragments. Since methods for creating gene libraries in *E. coli* are well established and random mutagenesis of the cloned material is straightforward, catalytic antibodies may then be screened directly on the bacterial colonies by their enzymic activity and/or binding. The relatively simple fermentation of *E. coli* makes large-scale production convenient, which would permit structure determination by both X-ray crystallography (Glockshuber et al 1990b) and NMR spectroscopy.

We have developed a system by which antibody fragments can be expressed in fully functional form in *E. coli* (Skerra & Plückthun 1988, Plückthun & Skerra 1989). This means that small fragments of antibodies can be produced directly without the need for proteolysis. The system was designed for the functional production of Fv fragments (the heterodimer of V_H and V_L; Fig. 1) or Fab fragments (the heterodimer of the whole light chain and the Fd fragment, which consists of the first two domains of the heavy chain). The crucial idea was the simultaneous secretion of both antibody chains into the periplasm, where the oxidizing environment allows the formation of disulphide bonds. Folding of either chain occurs in the presence of the other chain, giving correct assembly. The antibody fragments so produced can be purified by antigen affinity chromatography or immobilized metal ion chromatography (Skerra et al 1991) in a single step. This expression system has been extended to the expression in phage λ of an antibody library generated from an immunized animal (Huse et al 1989).

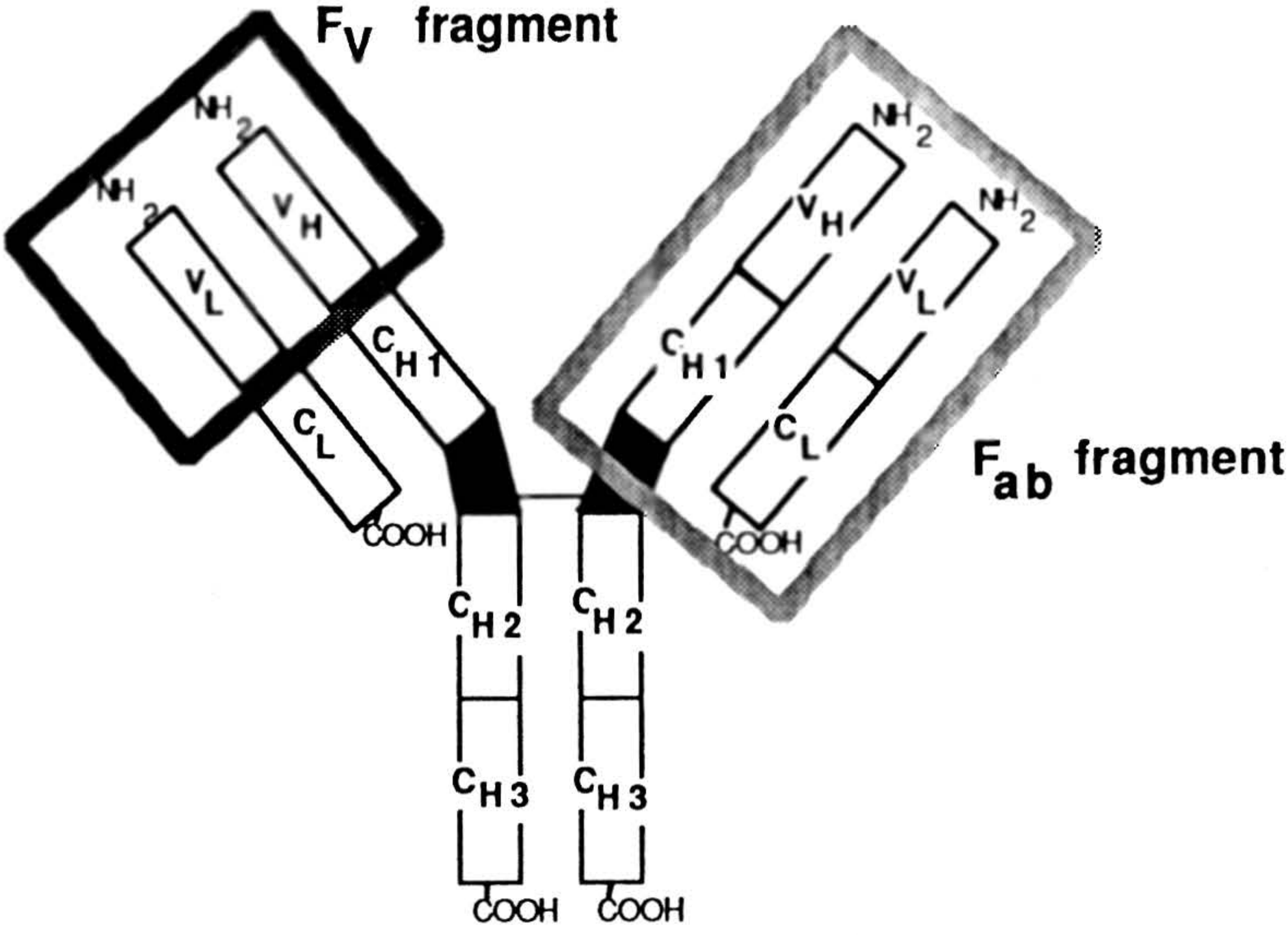

FIG. 1. The Fv and Fab fragments of an antibody, indicated in the schematic drawing of an immunoglobulin of the IgG class. The hinge region is shown in black.

The model system McPC603

Our experiments were carried out with the antibody McPC603, a phosphorylcholine-binding immunoglobulin A from the mouse. This antibody had been particularly well characterized: binding constants and kinetics (Leon & Young 1971, Metzger et al 1971, Young & Leon 1977, Goetze & Richards 1977a,b, 1978), sequences of both chains (Rudikoff & Potter 1974, Rudikoff et al 1981) and, most importantly, the three-dimensional structure are known (Segal et al 1974, Satow et al 1986). It was assumed from the three-dimensional structure that the antibody would catalyse a hydrolytic reaction (see below) (Plückthun et al 1987).

We have shown that the Fv fragment and the Fab fragment produced in *E. coli* have intrinsic hapten-binding constants indistinguishable from those of the whole antibody produced in mouse ascites or those of the proteolytic Fab′ fragment (Skerra & Plückthun 1988, Glockshuber et al 1990a, Skerra et al 1990). Thus our *E. coli* expression system produces fully functional antigen-binding fragments, and the Fv fragment has full antigen-binding capabilities. Before these fragments became conveniently accessible by our expression system, it was not clear whether Fv fragments would be functional (Inbar et al 1972, Sen & Beychok 1986).

At low protein concentrations the Fv fragment dissociates into V_H and V_L (Glockshuber et al 1990a), which leads to complicated hapten-binding behaviour.

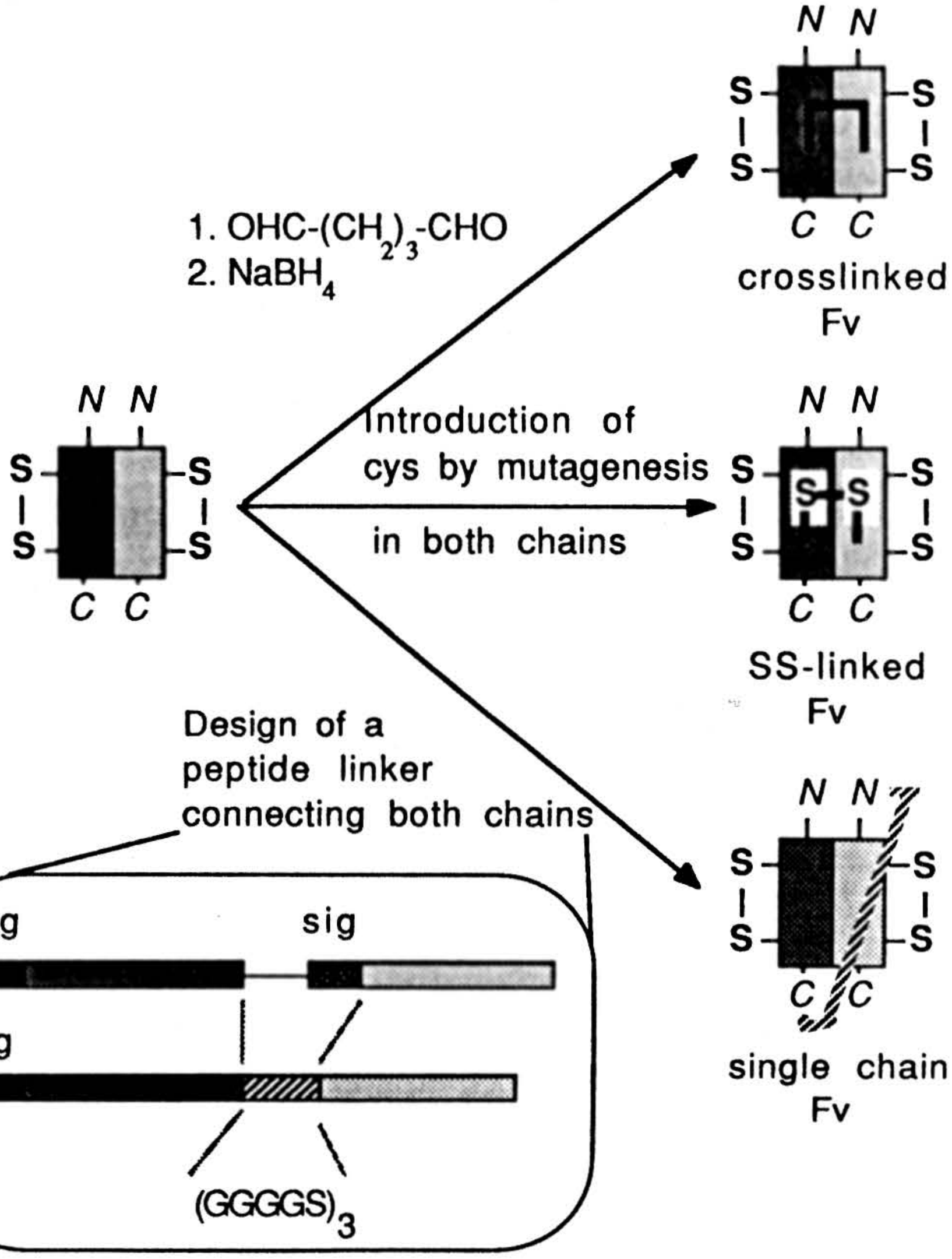

FIG. 2. Strategies designed to stabilize the association of the Fv fragment by linking the domains covalently. The top reaction arrow refers to chemical cross-linking with glutaraldehyde (1) and subsequent reduction (2). The middle arrow refers to stabilizing the Fv fragment by a new interchain disulphide bond, achieved by the introduction of cysteines by mutagenesis into both the heavy (V_H) and light (V_L) chains. The bottom arrow refers to the construction of a secreted single-chain antibody fragment, achieved by site-directed mutagenesis as shown for the genes on the bottom left. The intergenic region and the downstream signal sequence are replaced by a pentadecamer peptide linker $(GGGGS)_3$, which connects the C-terminus of the heavy chain to the N-terminus of the light chain.

We therefore devised three methods for stabilizing the fragment (Fig. 2). The first is chemical cross-linking with glutaraldehyde. The cross-linked Fv fragment shows hapten-binding constants identical to those of the Fab fragment under all conditions tested. Secondly, biochemical cross-linking was achieved by introducing an intermolecular disulphide bond between V_H and V_L at either of two positions (Glockshuber et al 1990a). These disulphide bonds form spontaneously *in vivo* and the resulting fragments have a hapten-binding affinity

almost identical to that of the whole antibody. A useful side effect of these modifications is a remarkably enhanced stability to thermal denaturation. Our third strategy was to construct a single-chain Fv fragment in which the two domains are joined covalently by a peptide linker. The single-chain Fv fragments that had been described previously (Bird et al 1988, Huston et al 1988) were expressed as inclusion bodies and refolded *in vitro*. In contrast, we added a signal sequence onto the single-chain Fv fragment and showed that it can then be transported, and normal functionality is obtained with the secreted single-chain fragment. Thus, three different strategies have been developed to solve the problem of chain dissociation in the Fv fragment without increasing its size. It should be noted, however, that the linking of the domains is not necessary, because the fully functional Fv fragment assembles by itself in the periplasm of *E. coli*.

An alternative to using the Fv fragment or the linked Fv fragment is to use the Fab fragment. This does not dissociate at low protein concentrations, because the two constant domains C_L and C_H1 contribute to stability. Interestingly, the yield of functional Fab fragment is consistently lower than that of the Fv fragment under identical conditions. A detailed analysis of this phenomenon (A. Skerra & A. Plückthun, in preparation) has shown that the reason is the lower efficiency of folding and assembly in the periplasm of *E. coli* and not a problem with expression, proteolysis, transport or processing.

Each of these fragments can thus be used for studies of catalysis; the particular applications envisaged will determine which considerations are most important and which fragments should be chosen for the experiment. We have used the Fv fragment in the studies described here, as it can be produced efficiently, and at the high protein concentration required in kinetic measurements dissociation is insignificant.

Catalysis by the recombinant Fv fragment of McPC603

The antibody McPC603 binds phosphorylcholine and esters of phosphorylcholine. Because phosphonates are well-known transition state analogues of peptide hydrolysis and efficient inhibitors of proteases of the metallo- and aspartyl types (Bartlett et al 1987, Kim & Lipscomb 1990), we expected that antibody McPC603 would also stabilize the tetrahedral intermediate of the hydrolysis of a choline-derived carboxylate ester. Similar ideas led Schultz's group to investigate the catalysis of hydrolysis of choline-*p*-nitrophenyl carbonate by the related antibodies T15 and M167 (Pollack et al 1986, Pollack & Schultz 1987). These antibodies are similar to McPC603 but their crystal structures have not yet been determined, and differences in the *length* of loops that contribute residues involved directly in phosphorylcholine binding make model building uncertain.

We investigated the catalysis of hydrolysis of choline-*p*-nitrophenyl carbonate by the Fv fragment of McPC603 expressed in *E. coli*. The kinetic constants

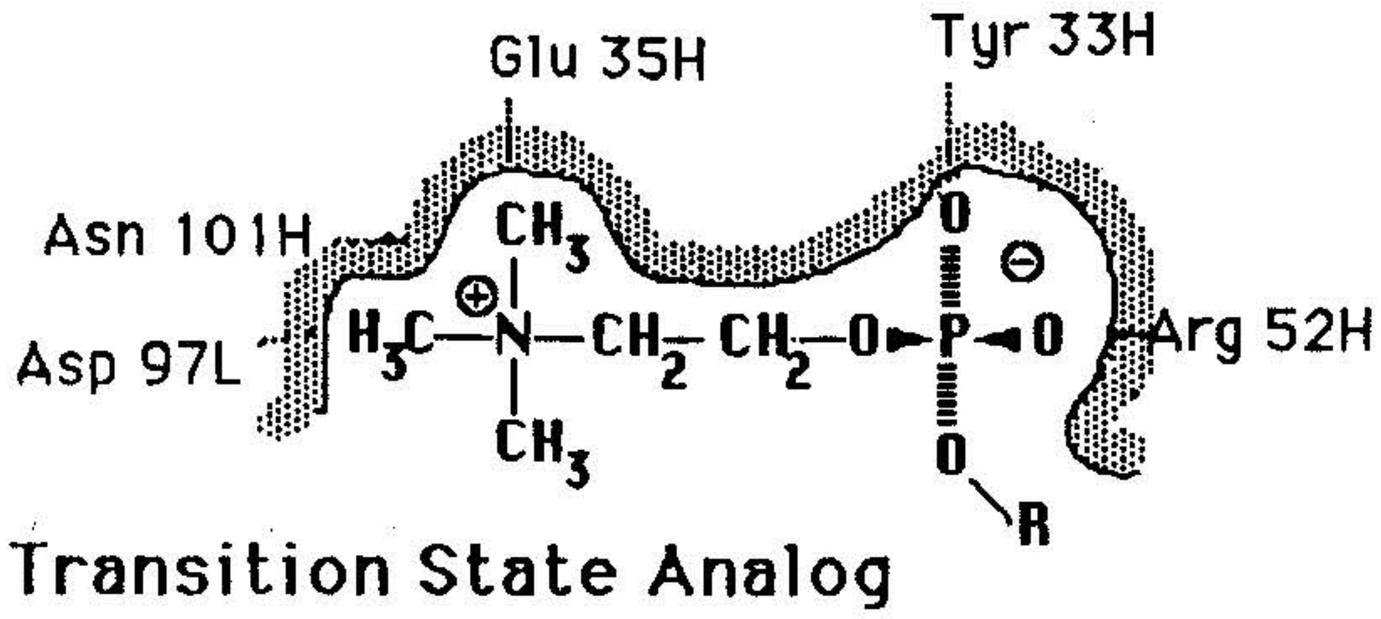

FIG. 3. Scheme for the binding of phosphorylcholine (top) and the transition state (or intermediate) in the hydrolysis of choline-*p*-nitrophenyl carbonate (R = *p*-nitrophenyl) by the active site of the antibody McPC603. The shaded area and marked residues constitute the antibody's binding site. The heavy chain residues Glu-35H, Tyr-33H, Arg-52H and Asn-101H and light chain residue Asp-97L were subjected to mutagenesis.

obtained ($k_{cat} = 0.045\ min^{-1}$ and $K_m = 1.3$ mM) are lower than those for the T15 and M167 antibodies produced from mouse ascites (Plückthun et al 1990). The hapten-binding constant for phosphorylcholine is identical for the Fv fragment from *E. coli* and the whole antibody, which suggests that the three-dimensional structure of the binding site does not change from antibody to fragment. We must therefore deduce that the difference in hydrolytic rate enhancement between McPC603 and M167 and T15 derives from slight differences in the structures of the binding sites of the different antibodies. Determination of the kinetic parameters of the Fv fragments of T15 and M167 produced in *E. coli* and of their mutants is in progress.

The pH dependence of the hydrolysis of choline-*p*-nitrophenyl carbonate catalysed by T15 and M167 (Pollack et al 1986, Pollack & Schultz 1987) suggests that no acid-base catalysis by the protein takes place. This is consistent with catalysis proceeding merely by transition state stabilization, which may be regarded as a kind of solvent effect. Therefore, the fine details of the binding site appear to determine the magnitude of the rate acceleration.

In a first set of experiments aimed at identifying the residues important for binding, we have mutated those which contact the phosphorylcholine directly (Fig. 3)

(Glockshuber et al 1991). The phosphate must interact with arginine and tyrosine; no other pair of residues tested at positions H33 and H52 functioned as well. The quaternary ammonium group's charge must be compensated by exactly one negative charge in position L97 or H101 (numbering according to the crystal structure of McPC603; Segal et al 1974, Satow et al 1986). No other charge pattern tested led to binding. We are determining the rates of hydrolysis catalysed by these and other mutants to improve our understanding of the relation between binding interactions and transition state stabilization.

Conclusion

Manipulation of the Fv fragment by protein engineering can now be done easily in the *E. coli* expression system that we have developed (Skerra & Plückthun 1988, Plückthun & Skerra 1989, Plückthun et al 1987, 1989). It extends the study of catalytic antibodies in several important ways. First, by using mutants of an antibody with a known structure, further insight into the structural requirements of binding and catalysis can be gained. Second, expression of the antibody in the native state in *E. coli* makes it possible to devise schemes for random mutagenesis, screening and even selection. Third, chemical catalysis in various forms can now be added to the binding site, thus combining various mechanistic devices in the same antibody binding site. Generation of catalytic antibodies by immunization combined with engineering and screening methods should lead to practically useful catalysts and a deeper understanding of enzyme catalysis.

References

Bartlett PA, Marlowe CK, Gianoussis PP, Hanson JE 1987 Phosphorus-containing peptide analogs as peptide inhibitors. Cold Spring Harbor Symp Quant Biol 52:83–90

Bird RE, Hardman KD, Jacobson JW et al 1988 Single-chain antigen-binding proteins. Science (Wash DC) 242:423–426

Boss MA, Kenten JH, Wood CR, Emtage JS 1984 Assembly of functional antibodies from immunoglobulin heavy and light chains synthesized in *E. coli*. Nucleic Acids Res 12:3791–3806

Brüggemann M, Neuberger MS 1988 Novel antibodies by DNA manipulation. Prog Allergy 45:91–105

Cabilly S, Riggs AD, Pande H et al 1984 Generation of antibody activity from immunoglobulin polypeptide chains produced in *Escherichia coli*. Proc Natl Acad Sci USA 81:3273–3277

Carter P, Wells JA 1988 Dissecting the catalytic triad of a serine protease. Nature (Lond) 332:564–568

Carter P, Wells JA 1990 Functional interaction among catalytic residues in subtilisin BPN'. Proteins Struct Funct Genet 7:335–342

Fersht A 1984 Enzyme structure and mechanism. W H Freeman, New York

Glockshuber R, Malia M, Pfitzinger I, Plückthun A 1990a A comparison of strategies to stabilize immunoglobulin F_v fragments. Biochemistry 29:1362–1367

Glockschuber R, Steipe B, Huber R, Plückthun A 1990b Crystallization and preliminary X-ray studies of the V_L domain of the antibody McPC603 produced in *E. coli*. J Mol Biol 213:613–615

Glockshuber R, Stadlmüller J, Plückthun A 1991 Mapping and modification of an antibody hapten binding site: a site-directed mutagenesis study of McPC603. Biochemistry, in press

Goetze AM, Richards JH 1977a Magnetic resonance studies of the binding site interactions between phosphorylcholine and specific mouse myeloma immunoglobulin. Biochemistry 16:228–232

Goetze AM, Richards JH 1977b Structure–function relations in phosphorylcholine-binding mouse myeloma proteins. Proc Natl Acad Sci USA 74:2109–2112

Goetze AM, Richards JH 1978 Molecular studies of subspecificity differences among phosphorylcholine-binding mouse myeloma antibodies using ^{31}P nuclear magnetic resonance. Biochemistry 17:1733–1739

Haldane JBS 1930 Enzymes. Longmans, Green, London; 1965 MIT Press, Cambridge, MA

Huse WD, Sastry L, Iverson SA et al 1989 Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. Science (Wash DC) 246:1275–1281

Huston JS, Levinson D, Mudgett-Hunter M et al 1988 Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain F_v analogue produced in *Escherichia coli*. Proc Natl Acad Sci USA 85:5879–5883

Inbar D, Hochman J, Givol D 1972 Localization of antibody-combining sites within the variable portions of heavy and light chains. Proc Natl Acad Sci USA 69:2659–2662

Iverson B, Lerner RA 1989 Sequence-specific peptide cleavage catalyzed by an antibody. Science (Wash DC) 243:1184–1188

Jencks WP 1969 Catalysis in chemistry and enzymology. McGraw-Hill, New York

Kim H, Lipscomb WN 1990 Crystal structure of the complex of carboxypeptidase A with a strongly bound phosphonate in a new crystalline form: comparison with structures of other complexes. Biochemistry 29:5546–5555

Kohen F, Hollander Z, Burd JF, Boguslaski RC 1979 A steroid immunoassay based on antibody-enhanced hydrolysis of a steroid-umbelliferone conjugate. FEBS (Fed Eur Biochem Soc) Lett 100:137–140

Kohen F, Kim JB, Barnard G, Lindner HR 1980 Antibody-enhanced hydrolysis of steroid esters. Biochim Biophys Acta 629:328–337

Köhler G, Milstein C 1975 Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (Lond) 256:495–497

Kraut J 1988 How do enzymes work? Science (Wash DC) 242:533–540

Leon MA, Young NM 1971 Specificity for phosphorylcholine of six murine myeloma proteins reactive with pneumococcus C polysaccharide and β-lipoprotein. Biochemistry 10:1424–1429

Metzger H, Chesebro B, Hadler NM, Lee J, Otchin N 1971 Modification of immunoglobulin combining sites. In: Amos B (ed) Progress in immunology (Proc 1st Int Congr Immunol), Academic Press, New York, p 253–267

Morrison SL, Oi VT 1989 Genetically engineered antibody molecules. Adv Immunol 44:65–92

Pauling L 1946 Molecular architecture and biological reactions. Chem Eng News 24:1375–1377

Plückthun A, Skerra A 1989 Expression of functional antibody F_v and F_{ab} fragments in *E. coli*. Methods Enzymol 178:497–515

Plückthun A, Glockshuber R, Pfitzinger I, Skerra A, Stadlmüller J 1987 Engineering of antibodies with a known three-dimensional structure. Cold Spring Harbor Symp Quant Biol 52:105–112

Plückthun A, Glockshuber R, Skerra A, Stadlmüller J 1990 Properties of F_v and F_{ab} fragments of the antibody McPC603 expressed in *E. coli*. Behring Inst Mitt 87:48–55

Pollack SJ, Schultz PG 1987 Antibody catalysis by transition state stabilization. Cold Spring Harbor Symp Quant Biol 52:97–104
Pollack SJ, Jacobs JW, Schultz PG 1986 Selective chemical catalysis by an antibody. Science (Wash DC) 234:1570–1573
Powell MJ, Hansen DE 1989 Catalytic antibodies—a new direction in enzyme design. Protein Eng 3:69–75
Raso V, Stollar BD 1975a The antibody–enzyme analogy. Characterization of antibodies to phosphopyridoxyltyrosine derivatives. Biochemistry 14:584–591
Raso V, Stollar BD 1975b The antibody–enzyme analogy. Comparison of enzymes and antibodies specific for phosphopyridoxyltyrosine. Biochemistry 14:591–599
Rudikoff S, Potter M 1974 Variable region sequence of the heavy chain from a phosphorylcholine binding myeloma protein. Biochemistry 13:4033–4038
Rudikoff S, Satow Y, Padlan E, Davies D, Potter M 1981 Kappa chain structure from a crystallized murine Fab: role of joining segment in hapten binding. Mol Immunol 18:705–711
Satow Y, Cohen GH, Padlan A, Davies DR 1986 Phosphocholine binding immunoglobulin Fab McPC603: an X-ray diffraction study at 2.7 Å. J Mol Biol 190:593–604
Schultz PG 1989 Antikörper als Katalysatoren. Angew Chem 101:1336–1348
Segal DM, Padlan EA, Cohen GH, Rudikoff S, Potter M, Davies DR 1974 The three-dimensional structure of a phosphorylcholine-binding mouse immunoglobulin Fab and the nature of the antigen binding site. Proc Natl Acad Sci USA 71:4298–4302
Sen J, Beychok S 1986 Proteolytic dissection of a hapten binding site. Proteins Struct Funct Genet 1:256–262
Shokat KM, Schultz PG 1990 Catalytic antibodies. Annu Rev Immunol 8:335–363
Skerra A, Plückthun A 1988 Assembly of a functional immunoglobulin F_v fragment in *Escherichia coli*. Science (Wash DC) 240:1038–1041
Skerra A, Glockshuber R, Plückthun A 1990 Structural features of the McPC603 Fab fragment not defined by the X-ray structure. FEBS Lett 271:203–206
Skerra A, Pfitzinger I, Plückthun A 1991 The functional expression of antibody Fv fragments in *Escherichia coli*: improved vectors and a generally applicable purification technique. Bio/technology 9:273–278
Slobin LI 1966 Preparation and some properties of antibodies with specificity toward p-nitrophenyl esters. Biochemistry 5:2836–2844
Walsh C 1979 Enzymatic reaction mechanisms. W H Freeman, New York
Wolfenden R 1976 Transition state analog inhibitors and enzyme catalysis. Annu Rev Biophys Bioeng 5:271–306
Young NM, Leon MA 1977 The binding of analogs of phosphorylcholine by the murine myeloma proteins McPC 603, MOPC 167 and S107. Immunochemistry 14:757–761

DISCUSSION

Schultz: When you measured binding to the transition state analogue, did you use nitrophenylphosphorylcholine or just phosphorylcholine?

Plückthun: We used phosphorylcholine, but we are now starting a series of experiments with substituted phenylphosphorylcholines.

Schultz: So you were comparing the binding of a substrate to the binding of a different transition state analogue, one with a dianion?

Plückthun: Yes; we have worried about that, which is why we are doing the experiments with nitrophenyl and other phenylphosphorylcholine derivatives. We have done one experiment in which hapten binding was compared with the binding to an affinity column. The ligand on the column was a diester, and therefore a mono-anion. Those mutants of antibody McPC603 for which we saw no binding to the column bound only very weakly to phosphorylcholine. From the one case we have analysed in more detail, which is a mutant with Tyr-33H changed to His, we estimate that failure to bind to the column means at least 1000-fold weaker binding to phosphorylcholine.

Schultz: It is surprising that substitution of Lys for Arg-52H resulted in an antibody with no binding affinity. We saw little change in a related Lys-52 mutant S107 antibody.

Plückthun: I can only speculate because we do not know the structure. There seem to be alternative binding modes for the hapten that may lead to the different behaviour of some of the mutants as well as of the different natural phosphorylcholine-binding antibodies. It may be that the steric requirements of position H52 are such that only arginine can fulfil them in McPC603.

Benkovic: When you say 'no binding', is that really no binding? What difference in affinity can you measure?

Plückthun: 'No binding' is the absence of binding to the affinity column. We have purified the His mutant independently; it has 1000-fold weaker binding and doesn't bind to the affinity column. The Phe mutant has a 60-fold weaker binding and binds to the affinity column. So 'no binding' must be a reduction of binding of between 60- and 1000-fold.

Benkovic: The value for the strength of the electrostatic interactions in your binding studies should be similar to those measured in enzyme mutagenesis experiments. I was really surprised when you said there was no binding, but if 'no binding' is 60–1000-fold weaker affinity, everything is consistent.

Arata: When you have a series of mutants of this antibody expressed in *E. coli*, how do you judge that each mutant has been properly refolded and has a correct tertiary structure?

Plückthun: The evidence is only circumstantial. We have detected the production of these proteins on Western blots; they are properly transported and processed. Some mutants, such as in the cysteine residues, are not made: those I described are not degraded, thus it is likely that they are folded correctly. We cannot rigorously distinguish whether there's a local structural reason for not binding to the affinity column or whether it's a general influence of the folding. That is what we would like to find out.

Arata: Is it possible to make circular dichroism measurements?

Plückthun: Yes, but I don't think circular dichroism measurements are sufficiently sensitive. It is not likely that there will suddenly be a mutation that transforms the antibody into a helix bundle.

Schultz: With regard to the point that a substrate may assume different orientations in the active site, we've carried out electron spin resonance experiments using spin-labelled haptens with phenyl phosphonate-specific antibodies. The spin label in many cases rotates freely, which suggests that in some of these antibodies the carbonyl group of the substrate can adopt many different orientations. There is some correlation between lack of catalytic activity and rotational freedom of the spin label.

Plückthun: I agree this is a likely interpretation. Clearly, we need to determine the preferred binding orientations.

Lerner: If that phenomenon were also true of enzymes, it would explain some of the requirement for holding tightly to the 'spinach' on the substrate that is not mechanistically interesting.

Jencks: That's entropy loss, which accelerates the reaction and *is* mechanistically interesting.

Hansen: Antibody McPC603 is catalytic for a carbonate substrate but not an ester substrate, is that correct?

Plückthun: We haven't done very careful studies on this question, but I think there is product inhibition by the acid from the ester substrate. We know that the acid product binds almost as well as does the phosphorylcholine and presumably there is just a single turnover.

Jencks: Can you make any generalizations about the interaction of the substrate with the binding site from your work in this system?

Plückthun: Binding is really a combination of shape and electrostatics, and shape really means hydrophobic interactions. It seems that even such a small substrate uses hydrophobic forces and that these hydrophobic interactions are essential. The real challenge is to understand this well enough to manipulate it constructively, and this is more difficult than some people believe.

Lerner: If you have a serine protease and study it to its finest detail, you probably understand how all serine proteases work. But if you have a diversity system with a large number of members, like the immune system, and study one member (antibody) to its finest detail, will you know anything about the next one?

Plückthun: I will know what I am aiming for, what my next transition state analogue should look like.

Lerner: Why would you know that?

Huse: If the transition state analogue is very similar to the previous one, then you will have some information. I agree that if it is different, you will have no useful information.

Plückthun: I doubt whether the purely immunological approach can make chymotrypsin.

Huse: So you think 'catalytic antibodies' is an oxymoron?

Plückthun: I think we will be able to obtain decent catalytic activities, but we won't obtain the equivalent of enzymes without using protein engineering.

Schultz: I am willing to bet a case of champagne that we can make a catalytic antibody that functions as well or better than the enzyme chorismate mutase within two years.

Lerner: I am willing to bet another case that the combination of factors, including metals, will approach the efficiency of chymotrypsin. For certain substrates, the nitroanilide catalytic antibody, NPN43C9, that Steve Benkovic has studied (this volume) is only a few orders of magnitude less efficient than chymotrypsin.

Plückthun: The example of metals illustrates exactly what I am saying—metals are put in by engineering. You can't immunize an animal such that the histidines are generated where you want them.

Lerner: That depends on the numbers. Secondly, you must immunize an animal to start with to give the substrate specificity. The rate of catalysis, to me, is not the intellectual heart of the matter.

Protein engineers would like, for example, to make lysozyme into trypsin. When you ask how they plan to do that they say, first I am going to take lysozyme and make it bind to an amide. After that I am going to make it catalytic. Using the immune system the first part is solved and that's the hard part. Extraordinarily high substrate specificity and discrimination between substrate and transition state are already programmable within the immune system to some extent. We don't approach the problem like a blind watchmaker who can't see the solution, but rather like a myopic watchmaker! We see the solution dimly and then use the immune system to refine it.

Plückthun: All of this would benefit from knowing, at least in a few cases, which structures of catalytic sites have worked and which haven't worked.

Lerner: No doubt, but we should not be too preoccupied with rate accelerations.

Jencks: Part of the problem is that we still cannot account very clearly for the catalytic activity of an ordinary enzyme. The distinction here is between complementary binding, which can be done quite well with antibody systems, and chemistry, which involves covalent bonds, general acid-base catalysis and so forth. The chemistry does not arise directly from the complementarity.

Lerner: But there is a certain inability to discriminate between binding and chemistry. If you protonate a leaving amine in an amide bond hydrolysis in a rate-limiting step, is that chemistry or binding? It is both.

Jencks: Chemistry usually requires different groups than does binding, although they can be the same.

Lerner: I do not dispute that we need a knowledge base. There are several X-ray structures of antigen–antibody complexes. Often, if there is a guanidinium in the antigen, there will be a carboxylate in the antibody.

Plückthun: From the organic chemist's point of view, if you use a guanidinium to bind a phosphate group, you would prefer bidentate binding. This is not what happens in the antibody McPC603; that is very clear from the electron

density. If one simply approaches this problem using general principles without having any detailed information, one is not going to make much progress.

Jencks: It's important to get some values for the energies of these interactions.

Lerner: The problem that Ian Wilson has, for example, is which crystal structure to solve. It is very different generating information in a diversity system and in a single protein which represents an evolutionary end-point.

Schultz: We have studied the phosphorylcholine-specific antibody, S107, and Andreas Plückthun has examined McPC603—they are quite homologous and yet we see completely different results with the Lys to Arg and Tyr to His mutations. So even for a family of related antibodies you can't generalize.

Wilson: For McPC603, one might assume that the charged groups were in approximately the same position in the antibody combining site, but this may not be the case.

Plückthun: The key point is that we don't want to make this assumption; we want to make observations. We need to obtain a different crystal form of the Fv fragment and try to get a structure of the mutants, as we have done for the light chains. For the light chains the structure determination has been very efficient—we could get a structure, including mutagenesis, purification, crystallization and refinement, within a few weeks.

Wilson: In antibody HyHEL5, if you substitute Arg-68 with Lys, you lose three orders of magnitude of binding avidity (Smith-Gill et al 1982).

Lerner: If you look at Allen Edmundson's light chain dimers, he claims that hydrophobic molecules go into the pocket and the flexible side chains are moved to wherever they need to be to accommodate binding.

Plückthun: We see this here as well. We have tried to model the light chain dimer based on the Bence-Jones protein REI, which is highly homologous to the light chain of McPC603. For the individual domains, the model was very good. For the orientation of the two domains with respect to each other, there was a small difference in the angle, even though the interfaces are almost identical. That is another variable that is very poorly understood.

Paul: Concerning the issue of diversity, antibodies with different amino acid sequences in their hypervariable segments can express similar binding specificities. Is it likely that catalytic mechanisms used by antibodies would show similar redundancy?

Huse: Are you asking whether there are more solutions to a particular catalytic reaction than to a binding reaction?

Paul: Enzymes appear to use a restricted set of mechanisms, and the structural demands for catalysis may be more stringent than those for binding. My intuitive feeling is that there would be fewer solutions for catalysis.

Plückthun: One has to define what one means by a solution. From a purely physical point of view, the solution would be an electric field around the substrate molecule with protons, point charges and field vectors in certain directions. In that case there are probably only very few solutions to catalytic mechanisms.

But there are many molecular arrangements or architectures that could create this electrostatic field with the protons and the hydrophobic interactions in the right place.

Hansen: It would be interesting to study carefully a reaction that is catalysed by a number of different antibodies to see how many solutions we have available.

Lerner: Both kinetically and physically. I grew up in the age of immunology when the histocompatibility system was just being discovered. I would go to meetings and people would describe the histocompatibility system molecule by molecule. Finally, one genius stood up and said, what's really interesting is that they are all different. We have to be very careful in the way we think about diversity systems rather than end-point evolutionary systems.

References

Benkovic SJ, Adams J, Janda KD, Lerner RA 1991 A catalytic antibody uses a multistep kinetic sequence. In: Catalytic antibodies. Wiley, Chichester (Ciba Found Symp 159) p 4–12

Smith-Gill SJ, Wilson AC, Potter M, Prager EM, Feldmann RJ, Mainhart CR 1982 Mapping the antigenic epitope for a monoclonal antibody against lysozyme. J Immunol 128:314–322