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

Engineering of Antibodies with a Known Three-dimensional Structure

A. PLÜCKTHUN, R. GLOCKSHUBER, I. PFITZINGER, A. SKERRA, AND J. STADLMÜLLER
Genzentrum der Universität München, c/o Max-Planck-Institut für Biochemie, D-8033 Martinsried, West Germany

There are several approaches to the study of catalysis. Most paradigms of enzymology are the fruit of the first approach, the continuous search for new enzymes and their judicious study. This "discovery" approach has led not only to the characterization of enzymes catalyzing new reaction types, but also to the meticulous dissection of the simplest elementary catalytic steps. A second approach, the modification of *one* enzyme by chemical or, nowadays, mainly genetic means (i.e., the perturbation of the catalytic machinery), has recently also begun to provide further insight into the mechanisms of catalysis (for review, see Leatherbarrow and Fersht 1986; Wetzel 1986; Knowles 1987). Most enzymes have probably evolved to a rather high efficiency. The consequence is that modification will, most probably, only reduce their activity or leave it unaffected when they are tested for the reaction and conditions for which they were intended by nature (Albery and Knowles 1977).

The third approach to the study of catalysis involves the "design" of suitable (probably at first rather modest) catalytic entities. The presumption here is that this may provide a very sensitive test of the requirements for efficient catalytic rate enhancements. Moreover, one may hope for the clearer delineation of such rules by a critical comparison of "professional" versus "home-built" catalytic entities. The success of all approaches depends on treating each new or mutant protein as a *new* enzyme to be thoroughly studied.

The principle of this design approach is, of course, by no means new. A multitude of model reactions have been devised for many classes of enzymatic reactions to probe the chemical feasibility of alternative pathways. Many detailed studies that included the binding aspect of catalysis were carried out on cyclodextrins, crown ether derivatives, and similar compounds (see, e.g., Cramer and Mackensen 1966; Bender and Komiyama 1978; Breslow et al. 1978; Tabushi et al. 1980; Breslow and Czarnik 1983; Hilvert and Breslow 1984; Cram and Trueblood 1985; Kellogg 1985; Breslow 1986; Lehn 1986; D'Souza and Bender 1987). The disadvantage of these otherwise elegant model systems is that they do not involve proteins as catalysts. The extrapolation to natural enzymes becomes therefore somewhat longer. In addition, the synthetic effort to produce them is not much diminished after a number of models have been successfully made. It was therefore thought advantageous to carry out investigations directly with a protein. That enzymes do not possess a *vis vitalis* is illustrated by occasional discoveries of serendipitous catalytic activities in proteins not destined to work as enzymes. Bovine serum albumin, for example, decomposes Meisenheimer complexes, whereas nitrophenyl-binding antibodies and even human serum albumin do not (Taylor and Vatz 1973). In addition, hemoglobin can act as a monooxygenase in a variety of reactions (Ferraiolo et al. 1984; Starke et al. 1984).

In search of a suitable protein with which to carry out such investigations on the engineering of active sites, we decided on antibodies. Antibodies comprise a family of rather stable and similar structures that are able to bind to a very large number of antigens. The essence of antibody structure has been well reviewed (Kabat 1978; Amzel and Poljak 1979; Davies and Metzger 1983). The antibody domains consist of a highly conserved framework of β sheets. The antigen-binding region is made up of six hypervariable loops (three derived from each chain) connecting the β strands within each subunit, and almost all binding interactions are contributed by residues from these loops.

Indeed, fortuitous catalytic (precisely: hydrolytic) activities have been discovered in antibodies as well. Slobin (1966) described the catalysis of the hydrolysis of nitrophenyl acetate, catalyzed in a bimolecular reaction by a base on the protein, but not related to the nitrophenyl-binding properties of the antibodies. Rather, he showed that in these antibodies, binding protected the substrate from hydrolysis. In later studies, specific yet serendipitous reactions were found. Some antibodies reacted only stoichiometrically (Kohen et al. 1980b) and others showed a sluggish turnover (Kohen et al. 1979, 1980a). These experiments demonstrated again that there is at least nothing in the antibody molecule that *prevents* a reaction from turning over in such a hypothetical active site.

One of the most essential features of enzyme catalysis is selective binding. Enzymes must distinguish their substrates from others, and they gain catalytic efficiency by differentiating substrate and product with respect to binding energies (Albery and Knowles 1977). Possibly the most effective contribution of binding to catalysis is, however, the further discrimination of the transition state as the structure to be bound most tightly (for review, see Albery and Knowles 1977; but see also Jencks 1975). This idea of transition state complementarity was already considered by Haldane (1930). Pauling (1946) expressed this concept succinctly and proposed the synthesis of transition state analogs (for re-

view, see Wolfenden 1976) as inhibitors. He had also recognized the fundamental similarity between enzymes and antibodies in binding specific molecules (Pauling 1948). The idea of actually using transition state analogs as haptens for immunization was probably first explicitly stated by Jencks (1969) and has been tested experimentally several times since then with varying degrees of success (Raso and Stollar 1975a,b; Tramontano et al. 1986a,b; Jacobs et al. 1987). We do not know whether it is possible, even in principle, to design transition state analogs that elicit optimal catalytic groups for many reactions in the antihapten antibody as opposed to eliciting just some way of holding on to the hapten.

As a starting point for investigations on binding and catalysis, we decided to use an antibody with a known three-dimensional structure that may be reasonably close to a transition-state-binding protein. Such a system is the phosphorylcholine-binding mouse immunoglobulin A McPC 603 (for reviews, see Potter 1977; Perlmutter et al. 1984). The structure (Segal et al. 1974) and sequence (Rudikoff and Potter 1974; Rudikoff et al. 1981) of this and many analogous proteins (Potter 1977; Kabat et al. 1983; Perlmutter et al. 1984) have been determined. In analyzing binding and catalysis by a macromolecule, we believe it is essential to have complete structural information available. Even though modeling of variants is necessary in this case as well, it remains on firmer ground. The availability of high-quality crystals for X-ray diffraction studies is still a major uncertainty for every new protein, and we therefore decided to choose a protein for which this hurdle has already been passed.

McPC 603 binds phosphorylcholine. Phosphorus compounds, such as phosphonates, phosphonamides, but also phosphate esters, have been recognized as good inhibitors of the enzymatic hydrolysis of suitable carboxyl esters and amides, and they conform to the criteria of transition state analogs (Weaver et al. 1977; Kam et al. 1979; Hofmann and Rottenberg 1980; Jacobsen and Bartlett 1981; Thorsett et al. 1982; Bartlett and Marlowe 1983, 1987; Galardy et al. 1983; Tronrud et al. 1986, 1987). The phosphorus provides all the ligands of the tetrahedral intermediate itself (which is thought to be structurally and energetically similar to the transition states leading to and away from it, by Hammond's principle). It does not require complexation with an enzyme nucleophile to yield the actual analog (as boronates or activated carbonyl compounds would to mimic the tetrahedral intermediate). It should therefore be effective in systems where the attacking nucleophile is not enzyme bound and can be displaced (i.e., where it is water). Indeed, all of the literature examples above concern the inhibition of Zn proteases. Since the antibody makes no specific contact to the bridging oxygen of phosphorylcholine, the choice of atoms around the carbonyl group in a substrate undergoing hydrolysis should be somewhat permissive.

While this work was in progress, Pollack et al. (1986) reported that a related antibody, MOPC 167, already had the spontaneous capability to accelerate the hydrolysis of a suitable carbonate ester. This observation underlined once again the validity of Pauling's concept formulated 40 years ago, and, in addition, demonstrated that the antibody chosen by us with a *known* three-dimensional structure may indeed provide a promising experimental model for the systematic study of binding and rate acceleration.

We therefore decided to work out an experimental system in which such an antibody may be altered by design and in which active fragments of the antibody and its mutants can be obtained in large amounts for experimental investigations. We describe here the gene synthesis and bacterial expression of the F_V and F_{ab} fragments of the antibody protein. The genes corresponding to the variable domains V_H and V_L were obtained synthetically using the known protein sequence (Rudikoff and Potter 1974; Rudikoff et al. 1981). The gene for the light-chain constant domain C_L was derived from a genomic clone (Altenberger et al. 1981), whereas the gene for the first constant domain of the heavy-chain C_{H1} was obtained from a cDNA clone of a mouse IgA1 (Auffray et al. 1981).

EXPERIMENTAL PROCEDURES

General methods. Bacterial growth was carried out according to the methods described by Miller (1972), and DNA manipulations were based on the procedures described by Maniatis et al. (1982). Plasmid isolation (Birnboim and Doly 1979; Holmes and Quigley 1981; Ish-Horowicz and Burke 1981), transformation of *Escherichia coli* (Dagert and Ehrlich 1979; Hanahan 1983), DNA sequencing (a variation of the method of Chen and Seeburg 1985), polyacrylamide gel electrophoresis of proteins (Laemmli 1970; Fling and Gregerson 1986), and Western blots (Blake et al. 1984) were carried out essentially as described previously.

DNA synthesis. The oligonucleotides were synthesized with an Applied Biosystems Model 380A synthesizer using the phosphoramidite method (Sinha et al. 1984). They were then purified by polyacrylamide gel electrophoresis, phosphorylated with polynucleotide kinase, hybridized, and ligated with T4 ligase using methodology similar to that described elsewhere (Dörper and Winnacker 1983; Rommens et al. 1983).

Affinity chromatography. A phosphorylcholine affinity ligand was synthesized, coupled to a column resin, and used as described elsewhere (Chesebro and Metzger 1972). The myeloma protein McPC 603 was a gift from M. Potter and was purified from ascites. IgG affinity chromatography of protein A fusion proteins was carried out as described elsewhere (Moks et al. 1987).

Protein purification. The purification of the insoluble LacZ fusion proteins was carried out in analogy to the procedure described by Nagai et al. (1985). The fusion protein was recovered from the insoluble part of

French press lysates, solubilized with 8 M urea, and separated from insoluble material by centrifugation. The fusion proteins become highly enriched by this procedure. The urea was removed by dialysis, and the material was subsequently cleaved with the blood-clotting protease factor Xa. The factor X was purified (Fujikawa et al. 1972a) from bovine blood and activated with Russell's viper venom protease to factor Xa as described elsewhere (Fujikawa et al. 1972b).

Cell fractionation. Cell-fractionation experiments were carried out essentially as described previously (Plückthun and Knowles 1987); the protein was not radioactively labeled but was instead detected by Western blotting (Blake et al. 1984).

RESULTS AND DISCUSSION

We chose to investigate the expression of the antibody genes in a bacterial host system because of the ease of genetic manipulations and the feasibility of rather rapid inexpensive fermentations. The present paper describes the expression of the F_V and F_{ab} fragments of the antibody McPC 603 in *E. coli*. Numerous studies on a whole range of antibodies have shown that only the F_{ab} portion is required for binding, and often even the F_V portion suffices (Hochman et al. 1976).

Four genes therefore had to be obtained: one for each of the variable domains (V_L and V_H) to express the F_V fragment alone and one each comprising V_LC_L and V_HC_H, respectively, thus making up the exact F_{ab} fragment that had been crystallized (Segal et al. 1974).

The genes for the F_V fragment were obtained synthetically. The design of the sequences according to the known protein sequences (Rudikoff and Potter 1974; Rudikoff et al. 1981) took the following points into consideration: (1) The number of unique restriction sites with sticky ends was maximized. (2) The RNA secondary structure was minimized iteratively, especially in the amino-terminal region. (3) Codons very rare in *E. coli* (Grosjean and Fiers 1982) were avoided. Otherwise, codon usage was not further restricted. The V_H gene was assembled from three subclones (*Eco*RI-*Xma*I, *Xma*I-*Pst*I, and *Pst*I-*Hin*dIII [at position 381]), each ligated from six oligonucleotides around 40–50 bases in length (Fig. 1A). The V_L gene was assembled from two subclones (*Eco*RI-*Sal*I and *Sal*I-*Hin*dIII [at position 360]), each ligated from eight oligonucleotides (Fig. 1B). The various subclones were tested and confirmed by DNA sequencing. Correct gene fragments were ligated, and the DNA sequences of the whole genes were again verified.

The genes for the F_{ab} fragment were obtained by fusing the synthetic genes to cloned genes coding for the constant domains. The V_HC_H gene (Fig. 1A) was obtained by linking a fragment from the cDNA clone of a mouse IgA with an identical C_{H1} sequence as in McPC 603 (Auffray et al. 1981), via synthetic oligonucleotides to the gene for the V_H domain. The corresponding construction of V_LC_L made use of a genomic κ light-chain C_L clone (Altenburger et al. 1981) that was linked to the synthetic gene for the V_L domain via synthetic oligonucleotides (Fig. 1B). Additionally, the carboxy-terminal ends of both genes had to be fitted with a *Hin*dIII site, and in the case of the heavy chain, a stop codon had to be introduced. This was achieved with oligonucleotide fragments and/or appropriate subcloning. The correctness of the DNA constructions was verified by DNA sequencing of both complete F_{ab} genes, i.e., V_HC_H and V_LC_L (Fig. 1). In addition, all four genes were brought under the control of bacterial promoters (e.g., *tac*), and in an in vitro translation experiment, it was demonstrated that the four proteins were of the expected sizes (V_L 115 amino acids; V_H 122 amino acids; V_LC_L 220 amino acids; V_HC_H 222 amino acids).

To aid in the detection of the proteins expressed in vivo, the genuine myeloma protein McPC 603 was purified from ascites by phosphorylcholine affinity chromatography, and antibodies against it were raised in a rabbit. Western blot experiments showed that these antibodies recognized the four recombinant proteins (V_L, V_H, V_LC_L, and V_HC_H) and that all four proteins could be expressed in vivo, albeit in moderate amounts (Fig. 2).

The expression of antibody genes in bacteria was reported previously (see, e.g., Boss et al. 1984; Cabilly et al. 1984; see also Boss and Wood 1985). In these and other experiments, cytoplasmic expression was intended and rather variable yields were reported. Refolding experiments led only to relatively low yields of recovered activity (Boss and Wood 1985). It must also be noted that the resulting proteins, when highly expressed in the cytoplasm, are likely to carry an uncleaved amino-terminal methionine or even a formylmethionine residue (Sherman et al. 1985). At present, it is not clear if and by how much this may affect the binding properties of an antibody. Preliminary evidence suggests that the variable domains of an immunoglobulin may be rather unstable in *E. coli*, and direct expression may be limited to larger fragments. Expression for preparative purposes was therefore investigated by three different methods: (1) expression of a highly expressed cytoplasmic hybrid protein with a protease-sensitive site, (2) expression as a potentially exportable hybrid protein with a protease-sensitive site, and (3) expression as a fusion with a signal sequence with ensuing transport to the periplasm.

In the first approach, fusions were constructed between a truncated β-galactosidase and the variable domains, linked by a recognition sequence for the blood-clotting protease factor Xa. These hybrid proteins were synthesized in high amounts and were found as inclusion bodies. They could therefore be highly enriched already by centrifugation of the cell lysate and urea extraction of the pellet (Fig. 3A). After solubilization, the hybrid protein was then cleaved with factor Xa, a protease of narrow specificity (Lottenberg et al. 1981). The cleavage mixture was fractionated by ion-exchange chromatography in the presence of a denaturant (8 M urea). By using this procedure, V_L could be obtained in pure form (Fig. 3B), whereas V_H could be obtained

A

EcoRI NcoI EcoRV Tth111I SacI PstI 80
GAATTCCATGGATATCGTTATGACCCAGTCTCCGAGCTCTCTGTCTGTATCTGCAGGTGAACGTGTTACCATGTCTTGCA
MetAspIleValMetThrGlnSerProSerSerLeuSerValSerAlaGlyGluArgValThrMetSerCys

BstEII BalI 160
AATCTTCTCAGTCTCTGCTGAACTCTGGTAACCAGAAAAACTTCCTGGCGTGGTATCAGCAAAAGCCTGGCCAACCGCCG
LysSerSerGlnSerLeuLeuAsnSerGlyAsnGlnLysAsnPheLeuAlaTrpTyrGlnGlnLysProGlyGlnProPro
========== CDR I ==========

SalI BspMII KpnI 240
AAACTGCTGATCTACGGTGCGTCGACCCGTGAATCTGGTGTTCCGGACCGTTTTACCGGTAGCGGTAGCGGTACCGACTT
LysLeuLeuIleTyrGlyAlaSerThrArgGluSerGlyValProAspArgPheThrGlySerGlySerGlyThrAspPhe
======== CDR II ========

BglII 320
CACTCTGACCATCTCTTCTGTACAGGCTGAAGATCTGGCTGTTTACTACTGTCAAAACGACCACTCTTACCCGCTGACCT
ThrLeuThrIleSerSerValGlnAlaGluAspLeuAlaValTyrTyrCysGlnAsnAspHisSerTyrProLeuThr
========= CDR III =========

NarI BssHII 400
TTGGCGCCGGCACCAAACTGGAACTGAAGCGCGCTGATGCTGCACCGACTGTATCCATCTTCCCACCATCCAGTGAGCAG
PheGlyAlaGlyThrLysLeuGluLeuLysArgAlaAspAlaAlaProThrValSerIlePheProProSerSerGluGln

HpaI 480
TTAACATCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAAGACATCAATGTCAAGTGGAAGATTGA
LeuThrSerGlyGlyAlaSerValValCysPheLeuAsnAsnPheTyrProLysAspIleAsnValLysTrpLysIleAsp

BclI 560
TGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTGATCAGGACAGCAAAGACAGCACCTACAGCATGAGCAGCA
GlySerGluArgGlnAsnGlyValLeuAsnSerTrpThrAspGlnAspSerLysAspSerThrTyrSerMetSerSer

640
CCCTCACGTTGACCAAGGACGAGTATGAACGACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTCACCC
ThrLeuThrLeuThrLysAspGluTyrGluArgHisAsnSerTyrThrCysGluAlaThrHisLysThrSerThrSerPro

DraII HindIII 711
ATTGTCAAGAGCTTCAACAGGAATGAGTGTTAGAGACAAAGGTCCTGATGCTGCTGATAGCAGGTAAGCTT
IleValLysSerPheAsnArgAsnGluCysEnd

B

EcoRI NcoI BamHI 80
GAATTCCATGGAAGTTAAACTGGTAGAGTCTGGTGGTGGTCTGGTACAGCCGGGTGGATCCCTGCGTCTGTCTTGCGCTA
MetGluValLysLeuValGluSerGlyGlyGlyLeuValGlnProGlyGlySerLeuArgLeuSerCysAla

SauI XmaI XhoI 160
CCTCAGGTTTCACCTTCTCTGACTTCTACATGGAGTGGGTACGTCAGCCCCCGGGTAAACGTCTCGAGTGGATCGCAGCT
ThrSerGlyPheThrPheSerAspPheTyrMetGluTrpValArgGlnProProGlyLysArgLeuGluTrpIleAlaAla
==== CDR I ====

NheI AccI HaeII SpeI 240
AGCCGTAACAAAGGTAACAAGTATACCACCGAATACAGCGCTTCTGTTAAAGGTCGTTTCATCGTTTCTCGTGACACTAG
SerArgAsnLysGlyAsnLysTyrThrThrGluTyrSerAlaSerValLysGlyArgPheIleValSerArgAspThrSer
========== CDR II ==========

ClaI PstI NsiI BssHII 320
TCAATCGATCCTGTACCTGCAGATGAATGCATTGCGTGCTGAAGACACCGCTATCTACTACTGCGCGCGTAACTACTATG
GlnSerIleLeuTyrLeuGlnMetAsnAlaLeuArgAlaGluAspThrAlaILeuyrTyrCysAlaArgAsnTyrTyr
=========

KpnI 400
GCAGCACTTGGTACTTCGACGTTTGGGGTGCAGGTACCACCGTTACCGTTTCTTCTGAATCTGCTCGTAACCCGACCATC
GlySerThrTrpTyrPheAspValTrpGlyAlaGlyThrThrValThrValSerSerGluSerAlaArgAsnProThrIle
=== CDR III ==========

NaeI AflII 480
TACCCGCTGACCCTGCCGCCGGCCTTAAGCTCTGACCCAGTGATAATCGGCTGCCTGATTCACGATTACTTCCCTTCCGG
TyrProLeuThrLeuProProAlaLeuSerSerAspProValIleIleGlyCysLeuIleHisAspTyrPheProSerGly

560
CACGATGAATGTGACCTGGGGAAAGAGTGGGAAGGATATAACCACCGTAAACTTCCCACCTGCCCTGGCCTCTGGGGGAC
ThrMetAsnValThrTrpGlyLysSerGlyLysAspILeuhrThrValAsnPheProProAlaLeuAlaSerGlyGly

HincII PvuII Bsp1286 640
GGTACACCATGAGCAACCAGTTGACCCTGCCAGCTGTCGAGTGCCCAGAAGGAGAATCCGTGAAATGTTCCGTGCAACAT
ArgTyrThrMetSerAsnGlnLeuThrLeuProAlaValGluCysProGluGlyGluSerValLysCysSerValGlnHis

FokI HindIII 693
GACTCTAACCCCGTCCAAGAATTGGATGTGAATTGCTGATAGCAGGTAAGCTT
AspSerAsnProValGlnGluLeuAspValAsnCysEndEnd

Figure 1. (*See facing page for legend.*)

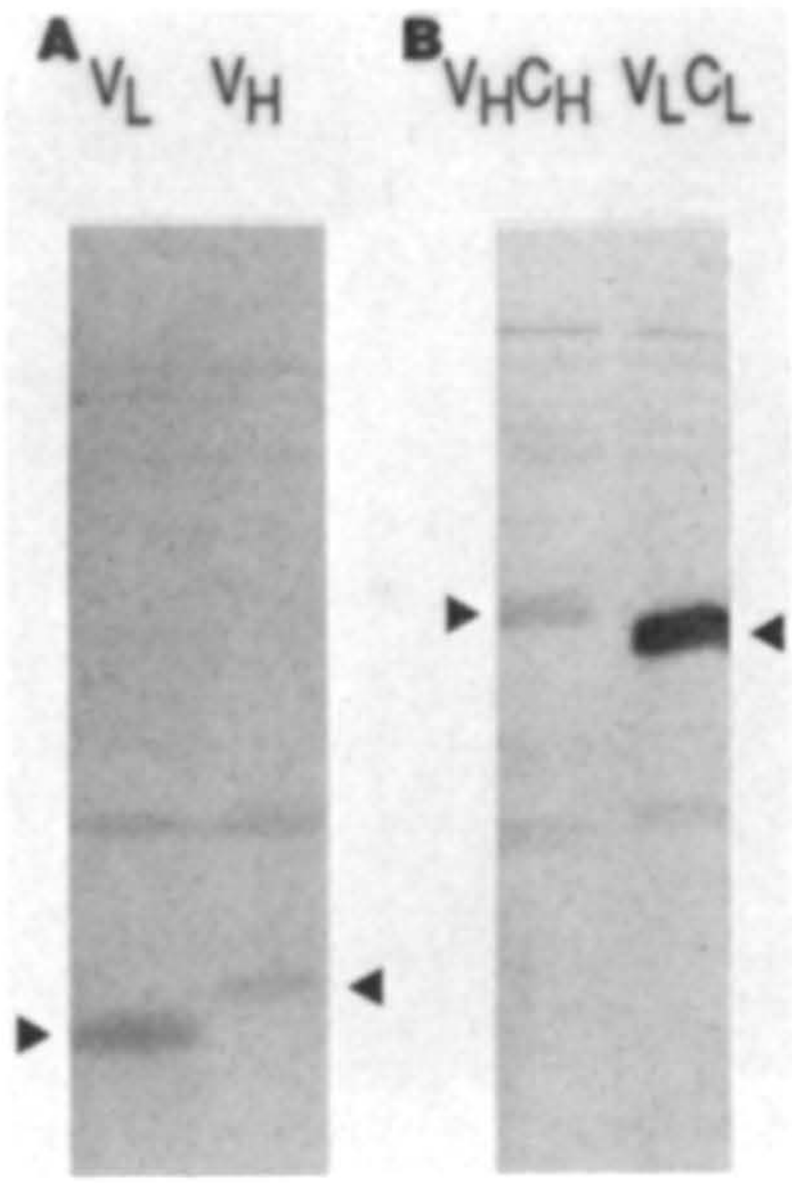

Figure 2. Expression of the proteins V_L, V_H, V_HC_H, and V_LC_L in *E. coli*. (*A*) Western blot of V_L and V_H expressed under the *trp* promoter in *E. coli* B. (*B*) Western blot of V_HC_H and V_LC_L under the control of the *tac* promoter in *E. coli* B.

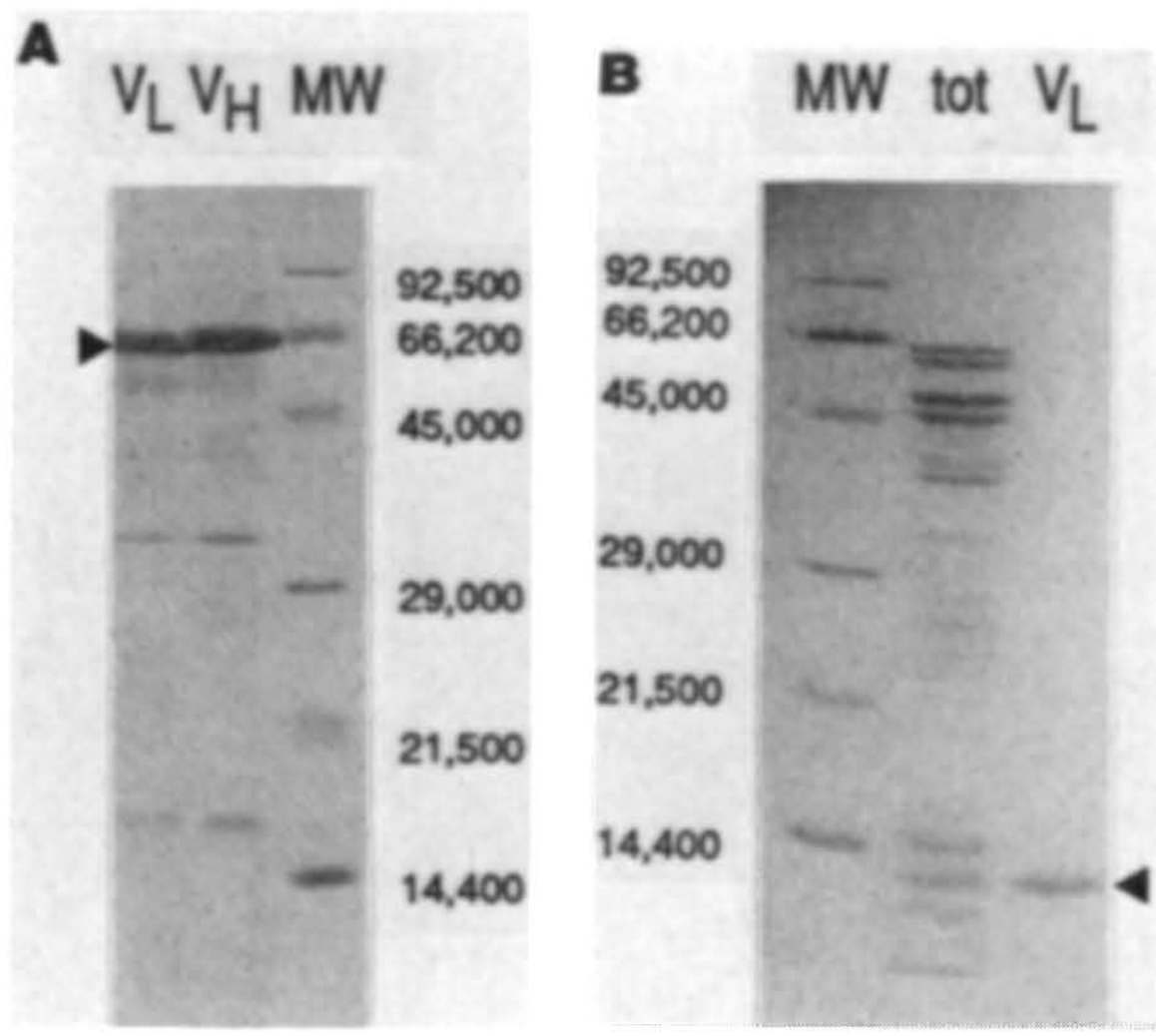

Figure 3. Purification of V_L and V_H. (*A*) Total insoluble protein from *E. coli* harboring *lacZ*-V domain fusions. V_L denotes the fusion of *lacZ* to V_L and V_H denotes the fusion of *lacZ* to V_H. (*B*) Purification of V_L from factor-Xa-cleaved fusion protein. *tot* denotes reaction mixture after partial factor Xa cleavage of fusion protein, and V_L denotes purified V_L protein. Polyacrylamide gels were stained with Coomassie blue.

highly enriched. It is now clear that the specificity of the blood-clotting factor Xa is somewhat more relaxed than originally thought (Nagai and Thogersen 1984) and it cuts within the truncated β-galactosidase as well as (very slowly) within V_H. The resulting variable-domain proteins were renatured by dilution from urea or guanidinium-HCl in the presence of reduced and oxidized glutathione, either each chain separately or both chains in a 1:1 mixture. Amino acid analyses for disulfide formation after iodoacetate derivatization of cysteines (Gurd 1967) suggested that the reoxidation and refolding yields found here have probably not surpassed reported values for antibodies.

In the second approach, fusions were constructed with domains of the *Staphylococcus aureus* protein A (Abrahmsen et al. 1985, 1986). This protein has been shown to be partially secreted to the culture medium of *E. coli* and to be able to direct the transport of a small peptide fused to the carboxyl terminus of protein A out to the culture medium. Protein A consists of one polypeptide chain with five homologous domains. We constructed a vector containing the first domain (called domain E) of protein A in tandem, fused to the variable domains V_H or V_L via a factor Xa recognition sequence. The cells expressed both hybrid proteins, and the V_L-containing fusion was found enriched in the growth medium. In fusions with the heavy chain, however, *E. coli* showed a severe growth defect and partial cell lysis; the plasmid is unstable. Therefore, further hybrid proteins were constructed, bringing the hybrid proteins under the control of the inducible *tac* promoter. These hybrid proteins contain only one domain E fused to either V_L, V_H, V_LC_L, or V_HC_H via a factor-Xa-sensitive site. These plasmids now remain stable when not induced. The fusions with V_H and V_L were further investigated, and it was shown that the hybrid proteins were partially transported to the medium with expression-dependent (i.e., inducible) partial cell lysis (Fig. 4). Only the mature form of the V_L fusion and small amounts of mostly the precursor form of the V_H fusion were found in the medium. The analysis of cytoplasmic, membrane, and periplasmic fractions (Plückthun and Knowles 1987) suggested that both the mature and precursor form are, to a large extent, found in the insoluble membrane fraction. That the mature size protein is indeed derived from transport through the membrane and subsequent signal peptidase cleavage is suggested by the inhibition of cleavage by the uncoupler carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) (Fig. 4) (Enequist et al. 1981). The CCCP sensitivity of maturation makes degradation by cytoplasmic proteases very unlikely. The precise understanding of the appearance of a band of mature size in the cytoplasmic and membrane fractions for both V_H and V_L fusions must await protease accessibility experiments (Minsky et al. 1986), which are in progress.

Figure 1. Nucleotide sequences of the V_LC_L gene (*A*) and of the V_HC_H gene (*B*). Many singular restriction sites are marked. The coding region of the V_L gene ends in alanine at position 355, followed by the sequence TGATAAGCTT, which codes for two stop codons and ends in a *Hin*dIII site. The coding region of the V_H gene ends in serine at position 376, followed by the same sequence TGATAAGCTT, which codes for two stop codons and ends in a *Hin*dIII site. Double underlines indicate the hypervariable loops (complementarity determining regions, CDR) according to the definition by Kabat et al. (1983).

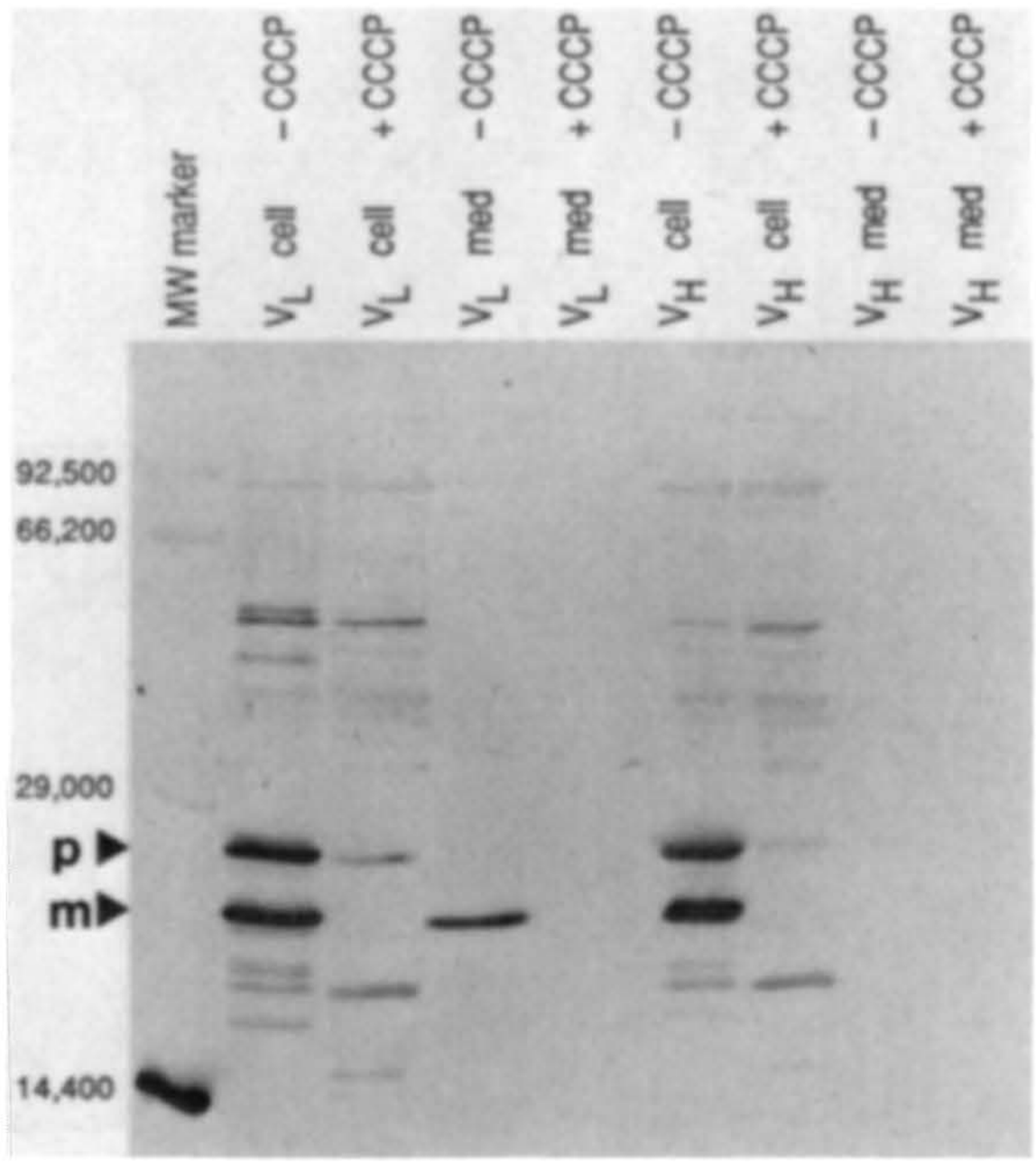

Figure 4. Sensitivity of the processing of protein A–variable domain fusions to the effect of the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). A Western blot of cellular and growth medium fractions of *E. coli* B is shown. + denotes samples treated with 0.1 mM CCCP as described in Results and Discussion and − denotes untreated. The precursor (p) and mature (m) forms of the hybrid proteins are indicated by arrowheads.

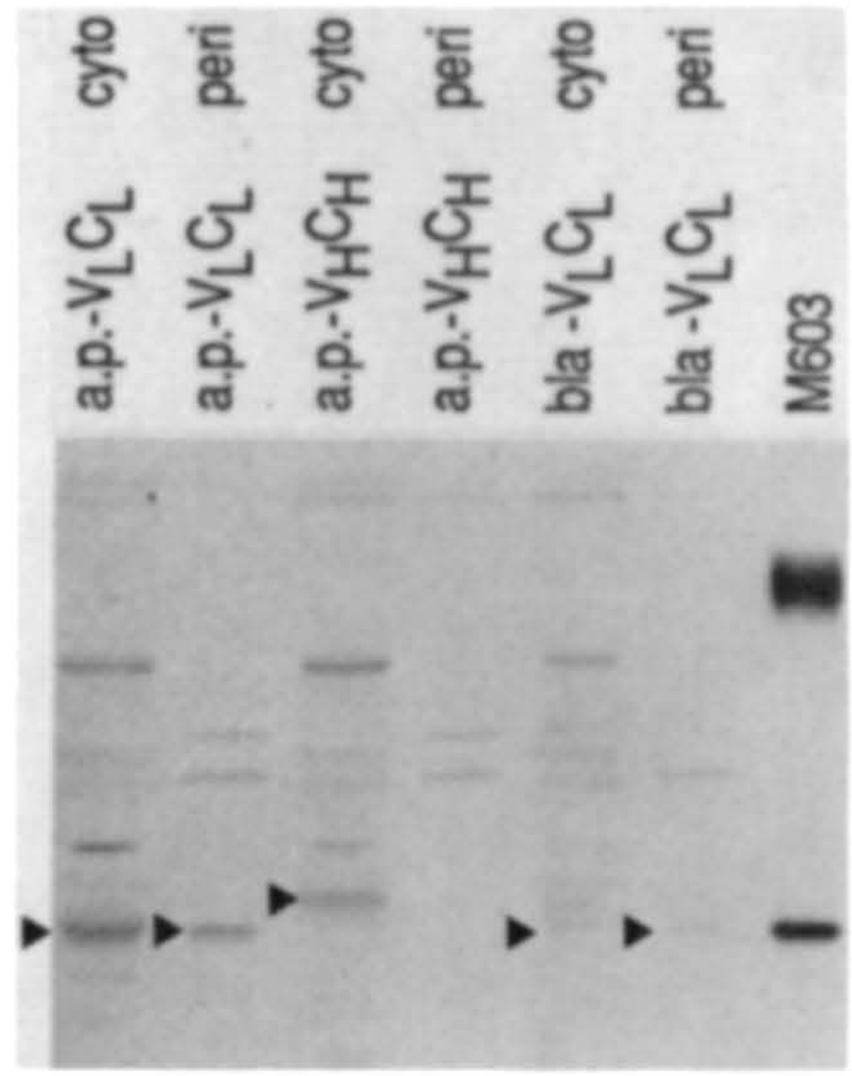

Figure 5. Cell fractionation of *E. coli* expressing V_LC_L or V_HC_H fused precisely to bacterial signal sequences. Western blot of periplasmic (peri) and cytoplasmic (cyto) fractions of fusions to alkaline phosphatase (a.p.) or β-lactamase (bla). M603 denotes the myeloma protein from ascites.

In the third approach, precise fusions were constructed between several signal sequences and V_L, V_H, V_LC_L, and V_HC_H. The transport to the bacterial periplasm is thought to result often in correct folding and in formation of correct disulfide bonds (for a recent review, see Briggs and Gierasch 1986). The antibody molecules are designed for export in eukaryotes via the endoplasmic reticulum. When V_LC_L and V_HC_H were fused precisely to the signal sequences of either alkaline phosphatase or β-lactamase, correct transport and cleavage were seen for the light chain. The heavy-chain fusion was cleaved, yet remained associated with the spheroblast fraction, i.e., was probably either membrane-associated or was released but remained insoluble (Fig. 5). We are now conducting experiments to elucidate the reason for this apparent difference in transport behavior between the two chains. It is conceivable that particular interactions between the light and heavy chain help the transport of the latter in the plasma cell endoplasmic reticulum or that the bacterial transport differs from the eukaryotic enough that the heterologous secretion and release are only possible for the light chain in bacteria, at least under the conditions tested. To eliminate the possibility that a particular interaction between the natural signal sequence and the protein may be critical, we also fused the natural eukaryotic signal sequence to the heavy chain. In this case, however, a very severe growth defect resulted that complicates the analysis of the fate of the protein.

The further optimization of these expression systems to very large scale preparations of protein remains an ongoing quest, since the full characterization of mutations will have to include structure determinations by X-rays of the mutant proteins. Such mutant antibody structures should aid substantially in the further improvement of methods for analyzing and predicting loop conformations.

The antibody domains have proved themselves throughout evolution as stable and suitable in numerous structures designed for particular versatility in recognition problems (realized, for example, in antibodies, MHC complexes, the T-cell receptor, as well as enzymes such as superoxide dismutase). They therefore provide very promising model systems with which to investigate the efficiency of binding ground states and transition states by an "engineering" approach.

ACKNOWLEDGMENTS

We express our gratitude to Dr. R. Mertz (Martinsried) for the synthesis of the oligonucleotides, to Dr. H. Zachau (Munich) for the clone of the mouse κ constant region, to Dr. F. Rougeon (Paris) for the clone of the IgA constant region, and to Dr. M. Potter (Bethesda) for mouse ascites.

REFERENCES

Abrahmsen, L., T. Moks, B. Nilsson, and M. Uhlen. 1986. Secretion of heterologous gene products to the culture medium of *Escherichia coli*. *Nucleic Acids Res.* **14:** 7487.

Abrahmsen, L., T. Moks, B. Nilsson, U. Hellman, and M. Uhlen. 1985. Analysis of signals for secretion in the staphylococcal protein A gene. *EMBO J.* **4:** 3901.

Albery, W.J. and J.R. Knowles. 1977. Efficiency and evolution of enzyme catalysis. *Angew. Chem. Int. Ed. Engl.* **16:** 285.

Altenburger, W., P.S. Neumaier, M. Steinmetz, and H.G. Zachau. 1981. DNA sequence of the constant gene region of the mouse immunoglobulin kappa chain. *Nucleic Acids Res.* **9:** 971.

Amzel, L.M. and R.J. Poljak. 1979. Three-dimensonal structure of immunoglobulins. *Annu. Rev. Biochem.* **48:** 961.

Auffray, C., R. Nageotte, J.L. Sikorav, O. Heidmann, and F. Rougeon. 1981. Mouse immunoglobulin A: Nucleotide sequence of the structural gene for the α-heavy chain derived from cloned cDNAs. *Gene* **13:** 365.

Bartlett, P.A. and C.K. Marlowe. 1983. Phosphonamidates as transition state analogue inhibitors of thermolysin. *Biochemistry* **22:** 4618.

———. 1987. Evaluation of intrinsic binding energy from a hydrogen bonding group in an enzyme inhibitor. *Science* **235:** 569.

Bender, M.C. and M. Komiyama. 1978. *Cyclodextrin chemistry*. Springer-Verlag, Berlin.

Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7:** 1513.

Blake, M.S., K.H. Johnston, G.J. Russel-Jones, and E.C. Gotschlich. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* **136:** 175.

Boss, M.A. and C.R. Wood. 1985. Genetically engineered antibodies. *Immunol. Today* **6:** 12.

Boss, M.A., J.H. Kenten, C.R. Wood, and J.S. Emtage. 1984. Assembly of functional antibodies from immunoglobulin heavy and light chains synthesized in *E. coli*. *Nucleic Acids Res.* **12:** 3791.

Breslow, R. 1986. Approaches to artificial enzymes. *Ann. N.Y. Acad. Sci.* **471:** 60.

Breslow, R. and A.W. Czarnik. 1983. Transaminations by pyridoxamine selectively attached at C-3 in β-cyclodextrin. *J. Am. Chem. Soc.* **105:** 1390.

Breslow, R., J.B. Doherty, G. Guillot, and C. Lipsey. 1978. β-Cyclodextrinbisimidazole, a model for ribonuclease. *J. Am. Chem. Soc.* **100:** 3227.

Briggs, M.S. and L.M. Gierasch. 1986. Molecular mechanisms of protein secretion: The role of the signal sequence. *Adv. Protein Chem.* **38:** 109.

Cabilly, S., A.D. Riggs, H. Pande, J.E. Shively, W.E. Holmes, M. Rey, L.J. Perry, R. Wetzel, and H.L. Heyneker. 1984. Generation of antibody activity from immunoglobulin polypeptide chains produced in *Escherchia coli*. *Proc. Natl. Acad. Sci.* **81:** 3273.

Chen, E.Y. and P.H. Seeburg. 1985. Supercoil sequencing: A fast and simple method for sequencing plasmid DNA. *DNA* **4:** 165.

Chesebro, B. and H. Metzger. 1972. Affinity labeling of a phosphorylcholine binding mouse myeloma protein. *Biochemistry* **11:** 766.

Cram, D.J. and K.N. Trueblood. 1985. Concept, structure and binding in complexation. In *Host guest complex chemistry: Macrocycles* (ed. F. Vögtle and E. Weber), p. 125. Springer-Verlag, Berlin.

Cramer, F. and G. Mackensen. 1966. A model for the action of chymotrypsin. *Angew. Chem. Int. Ed. Engl.* **5:** 601.

Dagert, M. and S.D. Ehrlich. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli*. *Gene* **6:** 23.

Davies, D.R. and H. Metzger. 1983. Structural basis of antibody function. *Annu. Rev. Immunol.* **1:** 87.

Dörper, T. and E.L. Winnacker. 1983. Improvements in the phosphoramidite procedure for the synthesis of oligodeoxyribonucleotides. *Nucleic Acids Res.* **11:** 2575.

D'Souza, V.T. and M.L. Bender. 1987. Miniature organic models of enzymes. *Accts. Chem. Res.* **20:** 146.

Enequist, H.G., T.R. Hirst, S. Harayama, S.J.S. Hardy, and L.L. Randall. 1981. Energy is required for maturation of exported proteins in *Escherichia coli*. *Eur. J. Biochem.* **116:** 227.

Ferraiolo, B.L., G. Onady, and J.J. Mieyal. 1984. Monooxygenase activity of human hemoglobin: Role of quaternary structure in the preponderant activity of the β-subunits within the tetramer. *Biochemistry* **23:** 5528.

Fling, S.P. and D.S. Gregerson. 1986. Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris-buffer system without urea. *Anal. Biochem.* **155:** 83.

Fujikawa, K., M.E. Legaz, and E.W. Davie. 1972a. Bovine factors X_1 and X_2 (Stuart factor). Isolation and characterization. *Biochemistry* **11:** 4882.

———. 1972b. Bovine factor X_1 (Stuart factor). Mechanism of activation by a protein from Russell's viper venom. *Biochemistry* **11:** 4892.

Galardy, R.E., V. Kontoyiannidou-Ostrem, and Z.P. Kortylewicz. 1983. Inhibition of angiotensin converting enzyme by phosphonic amides and phosphonic acids. *Biochemistry* **22:** 1990.

Grosjean, H. and W. Fiers. 1982. Preferential codon usage in prokaryotic genes: The optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes. *Gene* **18:** 199.

Gurd, F.R.N. 1967. Carboxymethylation. *Methods Enzymol.* **11:** 532.

Haldane, J.B.S. 1930. *Enzymes*. Longmans, Green and Co., London (1965, MIT Press, Cambridge, Massachusetts).

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166:** 557.

Hilvert, D. and R. Breslow. 1984. Functionalized cyclodextrins as holoenzyme mimics of thiamine-dependent enzymes. *Bioorg. Chem.* **12:** 206.

Hochman, J., M. Gavish, D. Inbar, and D. Givol. 1976. Folding and interaction of subunits at the antibody combining site. *Biochemistry* **15:** 2706.

Hofmann, W. and M. Rottenberg. 1980. A transition state analogous organophosphate inhibitor of carboxypeptidase A. In *Enzyme inhibitors* (ed. U. Brodbeck), p. 19. Verlag Chemie, Weinheim.

Holmes, D.S. and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114:** 193.

Ish-Horowicz, D. and J.F. Burke. 1981. Rapid and efficient cosmid cloning. *Nucleic Acids Res.* **9:** 2989.

Jacobs, J., P.G. Schultz, R. Sugasawara, and M. Powell. 1987. Catalytic antibodies. *J. Am. Chem. Soc.* **109:** 2174.

Jencks, W.P. 1969. Strain, distortion and conformation change. *Catalysis in chemistry and enzymology*, p. 282. McGraw-Hill, New York.

———. 1975. Binding energy, specificity and enzymic catalysis: The Circe effect. *Adv. Enzymol.* **43:** 219.

Jacobsen, N.E. and P.A. Bartlett. 1981. A phosphonamidate dipeptide analogue as an inhibitor of carboxypeptidase A. *J. Am. Chem. Soc.* **103:** 654.

Kabat, E.A. 1978. The structural basis of antibody complementarity. *Adv. Protein Chem.* **32:** 1.

Kabat, E.A., T.T. Wu, H. Bilofsky, M. Reid-Miller, and H. Perry. 1983. *Sequences of proteins of immunological interest.* U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, Maryland.

Kam, C.M., N. Nishino, and J.C. Powers. 1979. Inhibition of thermolysin and carboxypeptidase A by phosphoramidates. *Biochemistry* **18:** 3032.

Kellogg, R.M. 1985. Bioorganic modelling—Stereoselective reactions with chiral neutral ligand complexes as model systems for enzyme catalysis. In *Host guest complex chemistry: Macrocycles* (ed. F. Vögtle and E. Weber), p. 283. Springer-Verlag, Berlin.

Kohen, F., Z. Hollander, J.F. Burd, and R.C. Boguslaski. 1979. A steroid immunoassay based on antibody-enhanced

hydrolysis of a steroid-umbelliferone conjugate. *FEBS Lett.* **100:** 137.

Kohen, F., J.B. Kim, G. Barnard, and H.R. Lindner. 1980a. Antibody-enhanced hydrolysis of steroid esters. *Biochim. Biophys. Acta* **629:** 328.

Kohen, F., J.B. Kim, H.R. Lindner, Z. Eshhar, and B. Green. 1980b. Monoclonal immunoglobulin G augments hydrolysis of an ester of the homologous hapten. *FEBS Lett.* **111:** 427.

Knowles, J.R. 1987. Tinkering with enzymes: What are we learning? *Science* **236:** 1252.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680.

Leatherbarrow, R.J. and A.R. Fersht. 1986. Protein engineering. *Protein Eng.* **1:** 7.

Lehn, J.M. 1986. Recent studies of supramolecular catalysis and transport processes. *Ann. N.Y. Acad. Sci.* **471:** 41.

Lottenberg, R., V. Christensen, C.M. Jackson, and P.L. Coleman. 1981. Assay of coagulation proteases using peptide chromogenic and fluorogenic substrates. *Methods Enzymol.* **80:** 341.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.

Miller, J.H. 1972. *Experiments in molecular genetics.* Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.

Minsky, A., R. Summers, and J.R. Knowles. 1986. Secretion of β-lactamase into the periplasm of *Escherichia coli*: Evidence for a distinct release step associated with a conformational change. *Proc. Natl. Acad. Sci.* **83:** 4180.

Moks, T., L. Abrahmsen, B. Österlöf, S. Josephson, M. Östling, S.O. Enfors, I. Persson, B, Nilsson, and M. Uhlen. 1987. Large scale affinity purification of human insulin-like growth factor I from culture medium of *Escherichia coli*. *Biotechnology* **5:** 379.

Nagai, K. and H.C. Thogersen. 1984. Generation of β-globin by sequence-specific proteolysis of a hybrid protein produced in *E. coli*. *Nature* **309:** 810.

Nagai, K., M.F. Perutz, and C. Poyart. 1985. Oxygen binding properties of human mutant hemoglobins synthesized in *Escherichia coli*. *Proc. Natl. Acad. Sci.* **82:** 7253.

Pauling, L. 1946. Molecular architecture and biological reactions. *Chem. Eng. News.* **24:** 1375.

———. 1948. Antibodies and specific biological forces. *Endeavour* **7:** 43.

Perlmutter, R.M., S.T. Crews, R. Douglas, G. Sorensen, N. Johnson, N. Nivera, P. Gearhart, and L. Hood. 1984. The generation of diversity in phosphorylcholine-binding antibodies. *Adv. Immunol.* **35:** 1.

Plückthun, A. and J.R. Knowles. 1987. Consequences of stepwise deletions from the signal processing site in β-lactamase. *J. Biol. Chem.* **262:** 3951.

Pollack, S.J., J.W. Jacobs, and P.G. Schultz. 1986. Selective chemical catalysis by an antibody. *Science* **234:** 1570.

Potter, M. 1977. Antigen binding myeloma proteins of mice. *Adv. Immunol.* **25:** 141.

Raso, V. and B.D. Stollar. 1975a. The antibody-enzyme analogy. Characterization of antibodies to phosphopyridoxyl tyrosine derivatives. *Biochemistry* **14:** 584.

———. 1975b. The antibody-enzyme analogy. Comparison of enzymes and antibodies specific for phosphopyridoxyl tyrosine. *Biochemistry* **14:** 591.

Rommens, J.. D. MacKnight, L. Pomeroy-Cloney, and E. Jay. 1983. Gene expression: Chemical synthesis and molecular cloning of a bacteriophage T5 (T5P25) early promoter. *Nucleic Acids Res.* **11:** 5921.

Rudikoff, S. and M. Potter. 1974. Variable region sequence of the heavy chain from a phosphorylcholine binding myeloma protein. *Biochemistry* **13:** 4033.

Rudikoff, S., Y. Satow, E. Padlan, D. Davies, and M. Potter. 1981. Kappa chain structure from a crystallized murine F_{ab}: Role of joining segment in hapten binding. *Mol. Immunol.* **18:** 705.

Segal, D.M., E.A. Padlan, G.H. Cohen, S. Rudikoff, M. Potter, and D.R. Davies. 1974. The three-dimensional structure of a phosphorylcholine-binding mouse immunoglobulin F_{ab} and the nature of the antigen binding site. *Proc. Natl. Acad. Sci.* **71:** 4298.

Sherman, F., J.W. Stewart, and S. Tsunasawa. 1985. Methionine or not methionine at the beginning of a protein. *Bioessays* **3:** 27.

Sinha, N.D., J. Biernat, J. McManus, and H. Köster. 1984. Polymer support oligonucleotide synthesis. XVIII: Use of β-cyanoethyl-N,N-dialkylamino-/N-morpholino phosphoamidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. *Nucleic Acids Res.* **12:** 4539.

Slobin, L.I. 1966. Preparation and some properties of antibodies with specificity toward p-nitrophenyl esters. *Biochemistry* **5:** 2836.

Starke, D.W., K.S. Blisard, and J.J. Mieyal. 1984. Substrate specificity of the monooxygenase activity of hemoglobin. *Mol. Pharmacol.* **25:** 467.

Tabushi, I., Y. Kuroda, and A. Mochizuki. 1980. The first successful carbonic anhydrase model prepared through a new route to regiospecifically bifunctionalized cyclodextrin. *J. Am. Chem. Soc.* **102:** 1152.

Taylor, R.P. and J.B. Vatz. 1973. Bovine serum albumin as catalyst. Accelerated decomposition of a Meisenheimer complex. *J. Am. Chem. Soc.* **95:** 5819.

Thorsett, E.D., E.E. Harris, E.R. Peterson, W.J. Greenlee, A.A. Patchett, E.H. Ulm, and T.C. Vassil. 1982. Phosphorus-containing inhibitors of angiotensin-converting enzyme. *Proc. Natl. Acad. Sci.* **79:** 2176.

Tramontano, A., K.D. Janda, and R.A. Lerner. 1986a. Chemical reactivity at an antibody binding site elicited by mechanistic design of a synthetic antigen. *Proc. Natl. Acad. Sci.* **83:** 6736.

———. 1986b. Catalytic antibodies. *Science* **234:** 1566.

Tronrud, D.E., H.M. Holden, and B.W. Matthews. 1987. Structures of two thermolysin-inhibitor complexes that differ by a single hydrogen bond. *Science* **235:** 571.

Tronrud, D.E., A.F. Monzingo, and B.W. Matthews. 1986. Crystallographic structural analysis of phosphoramidates as inhibitors and transition-state analogs of thermolysin. *Eur. J. Biochem.* **157:** 261.

Weaver, L.H., W.R. Kester, and B.W. Matthews. 1977. A crystallographic study of the complex of phosphoramidon with thermolysin. A model for the presumed catalytic transition state and for the binding of extended substrates. *J. Mol. Biol.* **114:** 119.

Wetzel, R. 1986. What is protein engineering? *Protein Eng.* **1:** 3.

Wolfenden, R. 1976. Transition state analog inhibitors and enzyme catalysis. *Annu. Rev. Biophys. Bioeng.* **5:** 271.