JOURNAL OF MOLECULAR RECOGNITION, VOL. **10**, 169–181 (1997)

Recognition of *E. coli* Tryptophan Synthase by Single-Chain Fv Fragments: Comparison of PCR-Cloning Variants with the Parental Antibodies

François Brégégère,† Patrick England, Lisa Djavadi-Ohaniance and Hugues Bedouelle*
Unité de Biochimie Cellulaire (CNRS URA 1129), Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France

The use of a recombinant antibody fragment instead of a complete antibody, as a conformational probe for protein structure and folding studies, can be technically advantageous provided that the recombinant fragment and its parental antibody recognize the antigen through the same mechanism. Monoclonal antibodies mAb19 and mAb93 are directed against the $TrpB_2$ subunit of *Escherichia coli* tryptophan synthase and they have been extensively used as conformational probes of this protein. DNA sequences coding for single-chain variable fragments (scFv) of mAb19 and mAb93 were cloned and assembled by reverse transcription of the mRNAs from hybridomas and PCR amplification. A specialized plasmid vector, pFBX, was constructed; it enabled to express the scFvs as hybrids with the maltose-binding protein (MalE) in *E. coli,* and to purify them by affinity chromatography on cross-linked amylose. Six independent clones were sequenced for each hybridoma. All of them had differences in their nucleotide and amino acid sequences. A competition ELISA and the BIAcore™ biosensor apparatus were used to compare the energetics and kinetics with which the parental antibodies and the hybrids bound $TrpB_2$. The antigen binding properties of the hybrids were close to those of the parental antibodies and they were only weakly affected by the differences of sequence between the clones, with one exception. The stability of one of the hybrids and its antigen binding properties were strongly modified by a change of Gln6 into Glu, introduced into its V_H domain by the PCR primers. Simple models of bimolecular interaction did not fully account for the kinetic profiles obtained with the parental antibodies and the hybrids, and this complexity suggested the existence of a conformational heterogeneity in these molecules. © 1997 John Wiley & Sons, Ltd.

J. Mol. Recogn. **10**, 169–181 (1997) No. of Figures: 5 No. of Tables: 4 No. of References: 62.

Keywords: **antibody; BIAcore; fusion protein; hybrid protein; maltose-binding protein; library; recognition; single-chain Fv; variable fragment; tryptophan synthase.**

Received 20 September 1996; received 28 April 1997; accepted 29 April 1997

Introduction

During the past decade, monoclonal antibodies have been increasingly used as conformation-dependent reagents to investigate the structure of proteins, their conformational changes and their folding mechanisms. In particular, they have been used in kinetics experiments to identify and characterize the folding steps of a protein that involve the appearance of local native-like structures (Goldberg, 1991). Simultaneously, fast methods have been developed to clone the genes of Fv or Fab antibody fragments by PCR (Huse *et al*., 1989; Orlandi *et al*., Clackson *et al*., 1991), express them in *Escherichia coli* (Better *et al*., 1988; Skerra and Plückthun, 1988), and purify them independently of their antigen binding properties (Brégégère *et al*., 1994; Griffiths *et al*., 1994; Schier *et al*., 1995). Because the association between the variable domains, V_H and V_L, of Fv fragments can be unstable, they are often expressed as single polypeptide chains, or scFvs, in which the N-terminal end of one domain is fused with the C-terminal end of the other domain through a peptide link (Bird *et al*., 1989; Huston *et al*., 1988).

The use of monoclonal antibodies as conformational probes encounters two main technical difficulties. Firstly, one needs monovalent and homogeneous preparations of antibody fragments, Fv or Fab. Secondly, one needs a suitable method to monitor the association of the antibody fragment with the antigen in kinetics experiments, preferably a fluorescence signal (Goldberg, 1991). Recombinant antibody fragments, produced in *E. coli*, should help to solve these difficulties because in principle they are homogeneous and they can be engineered by site-directed mutagenesis to introduce fluorescent residues or residues to which a fluorescent group can be attached, within or close to the antigen binding site.

A critical issue for the replacement of monoclonal antibodies, produced in hybridomas, by recombinant Fvs or Fabs, produced in *E. coli*, as conformational probes, concerns the identity of their antigen-recognition properties. The 5′ and 3′ sequences of the recombinant genes are brought by the PCR primers which are used during their cloning and amplification. The sequences of these primers are usually mixed, degenerate or reached by consensus so that the N- and C-terminal sequences of the recombinant

* Correspondence to: H. Bedouelle. E-mail: hbedouel@pasteur.fr
† Current address: Myers Skin Laboratory, Institute of Life Sciences, The Hebrew University, Givat RaM, Jerusalem 91904, Israel.

 CCC 0952–3499/97/040169–13 $17.50

fragments and of the parental antibodies are not necessarily identical (Orlandi *et al*., 1989; Ward *et al*., 1989; Clackson *et al*., 1991; Nicholls *et al*., 1993; Zhou *et al*., 1994; Dattamajumdar *et al*., 1996). The processes of reverse transcription and amplification by PCR can introduce replication errors and thus sequence changes (Gopinathan *et al*., 1979; Eckert and Kunkel, 1991). The link between the V_H and V_L domains in an scFv fragment and the absence of a C_{H1} domain can perturb the conformation of the antigen-binding site (Whitlow *et al*., 1993; Desplancq *et al*., 1994; Mallender *et al*., 1996). The scFv molecules can sometimes form dimers and multimers (Griffiths *et al*., 1993; Desplancq *et al*., 1994; Whitlow *et al*., 1994). Finally, the fusion of an affinity handle could also perturb the recombinant fragment.

Monoclonal antibodies mAb19 and mAb93 have been extensively used to probe the conformational changes and the folding of the $TrpB_2$ subunit of *E. coli* tryptophan synthase (Goldberg, 1991). mAb19 has a continuous epitope. It binds the native form of $TrpB_2$ and a synthetic peptide, corresponding to residues 1 to 9 of TrpB, with similar affinities (Navon *et al*., 1995). In contrast, mAb93 binds to a non-continuous epitope, included within residues 340–397 of TrpB (Friguet *et al*., 1989a). The epitopes of mAb19 and mAb93 are distant in the crystal structure of tryptophan synthase (Hyde *et al*., 1988).

The aim of this work was to produce scFv fragments, derived from mAb19 and mAb93, in *E. coli* for their use as conformational probes. We constructed a phagemid vector, pFBX, that allows one to clone the rearranged variable genes of an antibody and to express them directly as a hybrid, scFv-MalE, between a scFv fragment and the maltose binding protein of *E. coli* (MalE), after their amplification and assembly by PCR. We cloned the variable genes of antibodies mAb19 and mAb93 in this vector, and compared the nucleotide sequences of 6 independent clones for each of them. We then produced the corresponding scFv-MalE hybrids in the periplasm of *E. coli* and purified them by affinity chromatography on cross-linked amylose, as MalE. We used a competitive ELISA and the BIAcore™ apparatus, based on surface plasmon resonance, to compare the energetics and kinetics with which the parental antibodies and the different variants of the hybrids bound $TrpB_2$. When combined with previous studies (Brégégère *et al*., 1994; England *et al*., 1997), the results validate MalE as a vector for antibody fragments of diverse specificities and provide us with an efficient way of producing, purifying and modifying monovalent antibody fragments for the needs of structural studies on $TrpB_2$.

Experimental

Strains, media and DNA techniques

Strains HB2200 (*recA*, *malT*) (Bedouelle and Duplay, 1988) and PD28 (*recA*, Δ*malE444, malT^{C}1*) (Duplay *et al*., 1987), plasmids pVD10 (Brégégère *et al*., 1994) and pTZ18R (Mead *et al*., 1986) have been described. Promoter *malEp* is fully silent in HB2200 and constitutive in PD28. The derivatives of HB2200 and PD28 that contained plasmids, were grown with LB or 2×YT medium (Difco) in the presence of 100 μg/ml ampicillin at 30°C unless otherwise indicated. The media were supplemented with 10 mg/ml glucose to repress the expressions of promoters *lacp* and *malEp*, and thus those of the scFv-MalE hybrids, and with 1 mg/ml glucose to optimally induce their expressions (Brégégère *et al*., 1994). Plasmid DNA was prepared by the method of alkaline lysis (Sambrook *et al*., 1989). Double-stranded DNA was sequenced using the T7 sequencing kit from Pharmacia-Biotech (Uppsala). Site-directed mutagenesis was performed as described (Kunkel *et al*. 1987).

Construction of plasmid pFBX

Plasmid pFBX was constructed by recombining the smaller *EcoRI-BamHI* fragment of plasmid pVD10 into the corresponding sites of plasmid pTZ18R. This DNA fragment carries the gene for a hybrid betweeen the scFv fragment of monoclonal antibody D1.3 and protein MalE, under the control of promoter *malEp* (Brégégère *et al*., 1994). Insertion of this fragment into pTZ18R puts *malEp* in tandem with the promoter *lacp*. We generated *SfiI*, *NcoI*, *NotI* and *KpnI* restriction sites in the recombinant plasmid by site-directed mutagenesis (Fig. 1). The *SfiI* site was introduced within the signal sequence of *malE*, close to its 3′-end, and the *NotI* site was introduced at the boundary between the V_L and MalE coding sequences. The positions of the *SfiI* and *NotI* sites in pFBX are compatible with those of plasmid pCANTAB6 (Recombinant Phage Antibody System, Pharmacia-Biotech). The introduction of the restriction sites changed five residues among seven in the C-terminal segment of the MalE signal peptide, and three residues among five in the linker pentapeptide between V_L and MalE. The nucleotide sequences of the mutated regions were checked in all the recombinant plasmids.

Cloning the scFv-coding sequences

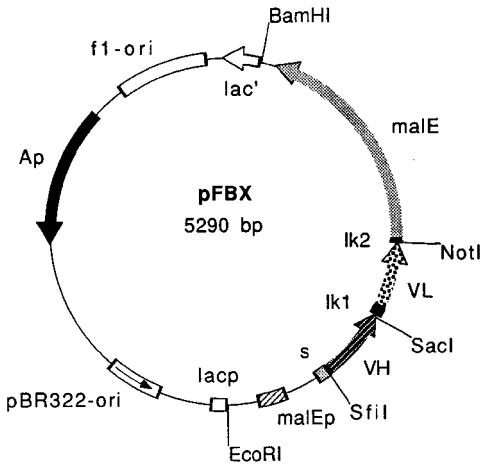
DNA fragments coding for the scFvs of antibodies mAb19 and mAb93 were obtained from the mRNAs of hybridoma cells as described (Orlandi *et al*., 1989; Clackson *et al*., 1991), using the Recombinant Phage Antibody System. Complementary DNAs, coding for V_H and V_L, were obtained by reverse transcription of the mRNAs. They were amplified using oligonucleotide primers which hybridized to the FR1 and FR4 regions of the rearranged variable genes, and 30 cycles of PCR (94°–55°–72° for mAb19; 94°–51°–72° for mAb93). The V_H and V_L coding sequences were linked with a central DNA segment, coding for $(Gly_4Ser)_3$, through seven cycles of PCR. The scFv coding sequence was finally amplified using 30 cycles of PCR and distal primers that contained *SfiI* and *NotI* restriction sites. The products of the final reaction were digested with *SfiI* and *NotI*, and ligated into the corresponding sites of plasmid pFBX. Strain HB2200 was transformed with the ligation products. Individual colonies of the transformants were grown in liquid medium, plasmid DNA was prepared and the preparations were used to transform strain PD28. Individual colonies of the HB2200 or PD28 transformants

were grown overnight at 30°C in liquid medium supplemented with 1 mg/ml glucose. Periplasmic extracts were prepared from these cultures and tested for anti-$TrpB_2$ activity by indirect ELISA, as described (Brégégère *et al*., 1994).

Purification and concentration of proteins

The scFv-MalE hybrids were produced from plasmid pFBX and its derivatives in strain PD28, extracted from the periplasm of the producing cells by a cold osmotic shock and purified by affinity chromatography on cross-linked amylose as described (Brégégère *et al*., 1994). The resulting preparations of the scFv-MalE hybrids were used for their functional characterization. From this point onwards, all the buffers used to further purify and to characterize the scFv-MalE hybrids, contained 1 mM maltose to prevent any dimerization of their MalE portion (Richarme, 1982). The partially purified preparations of the scFv-MalE hybrids were further fractionated by two additional steps of chromatography, performed with a Pharmacia FPLC system: an anion exchange chromatography, run with 30 mM Tris–HCl, pH 7.5, 1 mM maltose, and a gradient of 0 to 200 mM NaCl, in a MonoQ HR 5/5 column; and a gel filtration chromatography, run with 50 mM potassium phosphate, pH 7.0, 1 mM maltose, 150 mM NaCl, in a Superdex 200 HR 10/30 column, at a flow-rate of 0.5 ml/min. The following proteins were used as standards in the gel filtration: ferritin (molecular mass = 440 000, Stokes radius = 61.0 Å), aldolase (158 000, 48.1 Å) and bovine serum albumin (67 000, 35.5 Å). The concentration of protein in the preparations of the scFv-MalE hybrids were measured with the Biorad Protein Assay Kit (Hercules, California).

The purification of protein $TrpB_2$, the preparations of its holo-form (i.e. with bound pyridoxal 5-phosphate) and apo-form (i.e. without pyridoxal 5-phosphate) (Högberg-Raibaud and Goldberg, 1977; Zetina and Goldberg, 1982), and the purification of the monoclonal antibodies from ascitic fluids by ammonium sulfate precipitation and anion exchange chromatography (Friguet *et al*., 1989a; Larvor *et al*., 1994) were performed as described. The concentrations of apo-$TrpB_2$, of holo-$TrpB_2$ and of the antibodies in their purified preparations were calculated from the measures of A_{280nm}, using specific absorption coefficients equal to 0.58, 0.65 (Miles, 1970) and 1.5 ml/mg/cm (Onoue *et al*., 1965), respectively.




```
pVD10:      ... ATG ATG TTT TCC GCC TCG GCT CTC GCT / CAG GT ...
                 M   M   F   S   A   S   A   L   A / Q   V
pFBX:       ... ATG ATG GCG GCC CAG CCG GCC ATG GCT / CAG GT ...
                 M   M   A   A   Q   P   A   M   A / Q   V
                       MalE leader peptide           /  VH

pCANTAB5:   ... TTC TAT GCG GCC CAG CCG GCC ATG GCC / CAG GT ...
                 F   Y   A   A   Q   P   A   M   A / Q   V
                       PelB leader peptide           / linker DNA


pVD10:        ... AAA CGG / GCT GAT   GCT GCA GCT / AAA ATC ...
                   K   R  /  A   D     A   A   A  /  K   I
pFBX:         ... AAA CGG / GCG GCC   GCT GGT ACC / AAA ATC ...
                   K   R  /  A   A     A   G   T  /  K   I
                     VL   /    linker peptide     /  MalE

pCANTAB5:     ... AAA CGG   GCG GCC / GCA GAA ACT GTT GAA
                   K   R     A   A     A   E   T   V   E
                      linker DNA    /  fd gene 3 protein
```

Figure 1. pFBX, a phagemid for the expression of scFv-MalE hybrids. *Top:* partial restriction map of pFBX. The boxes represent open reading frames. *malEp* and *s* represent the promoter and the signal sequence of *malE*. *lk1* and *lk2* code for the peptide linkers $(Gly_4\text{-}Ser)_3$ and $Ala\text{-}Asp\text{-}Ala_3$, respectively. In the original plasmid, the V_H and V_L sequences were those of antibody D1.3. *Bottom:* sequence alignments of pVD10, pFBX and pCANTAB5 around the cloning sites. The *Sfi*I (GGCCCAGCCGGCC) and *Not*I (GCGGCCGC) sites, that are used for the insertion of the scFv-coding fragments, are underlined. The sites for *Nco*I (CCATGG) and for *Kpn*I (GGTACC) are in italics.

Measurements of the equilibrium and rate constants

We determined the equilibrium dissociation constants (K_D) between $TrpB_2$ and either the parental antibodies or the scFv-MalE hybrids by competition ELISA as described, except that we let the binding reactions equilibrate for 4 h at 4°C (Friguet *et al*., 1989b). This method does not require that the antibody preparation is pure. The rate constants for association and dissociation were measured with a BIAcore apparatus (Pharmacia-Biosensor). One partner (the ligand) was immobilized by covalently coupling its free amine groups to the carboxymethylated dextran surface of a CM5 sensor chip. The second partner (the analyte) was diluted in 10 mM phosphate buffer, pH 7.4, 2.7 mM KCl, 137 mM NaCl, 1 mM maltose and 0.05% Tween 20, and passed through the chip at a constant flow-rate of 5 μl/min at 20°C. After each experiment, the ligand binding sites were regenerated by flushing the chip with 10 μl of 15 mM HCl. We immobilized the antibody or the scFv-MalE molecules rather than the $TrpB_2$ molecules because $TrpB_2$ is formed by the non-covalent association of two subunits. The covalent coupling of $TrpB_2$ to the chip through only one subunit would not retain the other subunit when the chip is regenerated in denaturing conditions between two experiments. The native dimeric form of $TrpB_2$ would then be definitively lost. We used the dissociation constants of $TrpB_2$ into monomers, in the absence and in the presence of pyridoxal 5-phosphate, to calculate the actual molarities of the $TrpB_2$ dimers in the BIAcore experiments (Chaffotte and Goldberg, 1987; Wilson-Miles, 1991). These calculated

molarities were taken as antigen molarities in the input of the fitting models.

Analysis of the Biacore data

The kinetic data generated with the BIAcore apparatus were analysed by a non-linear least-square method (O'Shannessy *et al*., 1993), as implemented in the BIA-evaluation 2.1 software (Pharmacia-Biosensor, Uppsala). Exponential functions were fitted to the experimental data and the goodness of the fittings was evaluated with a χ^2 parameter. We first fitted the simple exponential function:

$$R=R_d*e^{-k_{off}*(t-t_d)} \quad (1)$$

to the profiles of dissociation of $TrpB_2$ from the ligand, where R is the BIAcore signal (proportional to the surface concentration of protein on the sensor chip), t is time, R_d and t_d are the values of R and t at the start of the measures, and k_{off} is a fitting parameter.

Similarly, we fitted the function:

$$R=k_0/k_{obs}*(1-e^{-k_{obs}*(t-t_a)})+R_a \quad (2)$$

to the profiles of association of $TrpB_2$ to the ligand, where t_a is the start time of the association, and R_a, k_0 and k_{obs} are fitting parameters. In the case of a reversible association between two homogeneous populations of analyte and ligand molecules to give a homogeneous binary complex, k_{off} is the dissociation rate constant of the process, k_{obs} is the apparent association rate constant and k_0 is the initial binding rate. k_{obs} and k_{off} are then linked by the relationship:

$$k_{obs}=k_{on}*C+k_{off} \quad (3)$$

where k_{on} is the association rate constant of the process and C is the analyte concentration. Practically, k_{on} can be determined by fitting a linear function to the related values of k_{obs} and C, and

$$K'_D=k_{off}/k_{on} \quad (4)$$

is the equilibrium dissociation constant of the complex between the analyte and the immobilized ligand. We call 'simple mechanism', this first method of analysis.

We also analysed the dissociation and association profiles with sums of two exponentials to improve the quality of the fittings:

$$R=R_1*e^{-k_{off1}*(t-t_d)}+(R_d-R_1)*e^{-k_{off2}*(t-t_d)} \quad (5)$$

$$R=Req_1*(1-e^{-k_{obs1}*(t-t_a)})+Req_2*(1-e^{-k_{obs2}*(t-t_a)})+R_a \quad (6)$$

where R, R_d, t, t_d and t_a are like in (1) and (2), and k_{off1}, k_{off2}, R_1, k_{obs1}, k_{obs2}, R_a, Req_1 and Req_2 are fitting parameters. In the case of a reversible association between a homogeneous analyte and two distinct populations of ligand molecules giving two binary complexes, k_{off1} and k_{obs1} on the one hand, and k_{off2} and k_{obs2} on the other hand represent partial rate constants and are linked by linear relationships similar to (3). R_1 is the portion of R_d that is attributable to one of the complexes, Req_1 and Req_2 represent the partial signal increases, at steady state, corresponding to the formation of the two complexes. Two partial association rate constants, k_{on1} and k_{on2}, and two equilibrium dissociation constants, K_{D1}' and K_{D2}', can be determined as above. We call 'dual mechanism' this second method of analysis.

Results

Cloning and expression of variable genes as scFv-MalE hybrids

We used plasmid pFBX as a vector to clone the variable genes of antibodies mAb19 and mAb93, and to express them as hybrid proteins, scFv-MalE, between single chain variable fragments and protein MalE. We obtained complementary DNAs, coding for the V_H and V_L domains of each antibody, by reverse transcription of the mRNAs from hybridomas, and we assembled them into a unique gene by PCR. We then inserted the gene that resulted from this assemblage and coded for a scFv, within the *malE* gene, immediately downstream from its signal sequence. The resulting fusion gene was under the control of promoter *malEp* and coded for scFv-MalE (Fig. 1).

We initially introduced the recombinant plasmids into HB2200 because *malEp* is inactive in this strain, which avoids a counterselection of the genes that code for toxic proteins. Yet, we detected some expression of the scFv-MalE hybrids in the HB2200 transformants (see below). We presumed that the cloned genes were transcribed from promoter *lacp*, which is located immediately upstream from *malEp* in pFBX and its derivatives. This low level of expression was not toxic at 30°C and allowed us to screen the HB2200 transformants directly, by assaying their periplasmic extracts for anti-$TrpB_2$ activity in ELISA. We tested 36 independent bacterial clones for each antibody. Four clones derived from mAb19 and three clones from mAb93 gave strong positive signals in ELISA (about 10% of the clones). Four clones derived from mAb19 and three clones from mAb93 gave weak signals (10%). The other clones (80%) were negative in the assay.

We also tested the capacity of the recombinant plasmids to express an scFv-MalE protein. Firstly, we took independent HB2200 transformants at random, prepared their plasmid DNAs, introduced these DNAs into strain PD28 and analysed periplasmic extracts of the recombinant PD28 by Western experiments, using an anti-MalE serum. Half of the 24 plasmids tested (12 for each antibody) did not express any detectable amount of MalE antigen in PD28 and therefore likely contained a non-sense mutation in their scFv coding sequence. Secondly, we repeated these experiments with the HB2200 transformants that gave positive signals in ELISA. Most of the corresponding plasmids expressed a protein species that corresponded to a full-length hybrid and was in variable amount, and other species that had apparent molecular masses between 40 000 and 50 000 and corresponded likely to incomplete products (data not shown).

Sequence differences between independent plasmid clones

The differences of properties between the plasmid clones came most probably from cloning artefacts. They posed the following problems. Which plasmid clones carried a sequence representative of the parental antibody? Could one reconstruct the parental sequence from the different clones? Did the different responses in ELISA come from variations in the levels of expression of the scFv-MalE hybrids or in their affinity for the antigen? Which clones expressed the scFv-MalE hybrids with the properties closest to the parental antibody? To answer these questions, we further characterized, structurally and functionally, three plasmid clones that gave strong signals during the initial screening by ELISA, and three clones that gave weak signals, for each of mAb19 and mAb93. We first determined the coding sequences of the whole scFv genes. We observed differences between independent clones but we could nevertheless establish a consensus sequence for each antibody (Tables 1 and 2; Fig. 2). We found a total of 29 nucleotide and 12 amino acid differences in the six sequences derived from mAb19, 15 nucleotide and four amino acid differences in the six sequences derived from mAb93. Among the 44 nucleotide differences, 35 were concentrated at the ends of the variable genes, in codons H2-H8, H104–H110, L4–L9 and L101–L106, which overlapped the PCR primers; four belonged to the link between the V_H and V_L coding sequences of mAb19; and five were scattered along the remainder of the coding sequences. Among these five, only three led to amino acid changes: one in the FR1 region of V_H and one at the junction between the CDR2 and FR3 regions of V_L in the case of mAb19; and one in the CDR2 of V_H in the case of mAb93 (Tables 1 and 2).

Purification of the scFv-MalE hybrids

We produced large amounts of the scFv-MalE hybrids in strain PD28, under control of promoter *malEp* which is constitutive in this strain, and we purified them by affinity chromatography on cross-linked amylose. We used the preparations that resulted from this first step of purification, for the functional characterization of the scFv-MalE hybrids (see below). We chose to further purify and analyse the preparations of two variants, Hyb19-22 and Hyb93-11, because they corresponded to clones that gave strong signals during the initial screening by ELISA. After the column of cross-linked amylose, the preparations of Hyb19-22 and Hyb93-11 were 90 and 40% pure, respectively, and

Table 1. Sequence variations in the clones derived from mAb19

Position	Region	Codon	19-04	19-12	19-20	19-22	19-24	19-27	a.a. change
H2	FR1	GTG			GTC			GTC	no
H3	FR1	AAG				AAA	AAA		no
H3	FR1	AAG	CAG		CAG				K→Q
H6	FR1	CAG			GAG				Q→E
H7	FR1	TCT	TCA						no
H8	FR1	GGG					AGG		G→R
H19	FR1	AAG		AGG					K→R
H49	FR2	GGA			GGG				no
H104	FR4	GGC			GGG				no
H105	FR4	CAA		CAG					no
H106	FR4	GGC						GGG	no
H110	FR4	ACC			ATC				T→I
K1	Linker	GGT	TGT						G→C
K6	Linker	GGC			TGC				G→C
K13	Linker	GGC						GGG	no
K13	Linker	GGC					TGC		G→C
L4	FR1	CTC			ATG				L→M
L5	FR1	ACT		ACC	ACC				no
L8	FR1	CCA				CCG			no
L9	FR1	GCC						ACC	A→T
L24	CDR1	CGA	CGG						no
L57	FR3	GGT		GAT					G→D
L101	FR4	GGG					GGC		no
L102	FR4	ACC	ACA					ACA	no
L104	FR4	CTG	TTG					TTG	no
L105	FR4	GAA			GAG	GAG			no
L106	FR4	ATA						ATC	no
L106	FR4	ATA			CTG	CTG			I→L

The table lists the codons of the scFv-MalE hybrids that were not conserved in the 6 sequenced plasmid clones. The codons are numbered and assigned to framework (FR) or complementarity-determining regions (CDR) according to Kabat *et al.* (1991). They are prefixed with 'H' for heavy-chain and 'L' for light-chain. The codons of the peptide linker between V_H and V_L are numbered separately and prefixed with 'K'. The third column gives the consensus (i.e. majority) codons. The following columns give the nucleotide changes relative to the consensus for each sequenced clone. The last column gives the corresponding amino acid changes. The complete sequence of the consensus is given in Fig. 3.

Table 2. Sequence variations in the clones derived from mAb93

Position	Region	Codon	93-01	93-10	93-11	93-29	93-31	93-34	a.a. change
H2	FR1	GTC				GTG	GTG		no
H3	FR1	AAG				CAG		CAG	K→Q
H3	FR1	AAG	GAG						K→E
H6	FR1	CAG					GAG	GAG	Q→E
H7	FR1	TCT		TCA				TCA	no
H8	FR1	GGA			GGG				no
H64	CDR2	AAG						AGG	K→R
H106	FR4	GGG		GGC	GGC			GGC	no
L5	FR1	ACT	ACC						no
L6	FR1	CAG			CAA				no
L6	FR1	CAG		TAG					STOP
L8	FR1	CCA			CCT				no
L101	FR4	GGG		GGC			GGC		no
L102	FR4	ACA		ACC		ACC	ACC		no
L104	FR4	TTG		CTG		CTG	CTG		no
L106	FR4	ATA		ATC		ATC	ATC		no

See footnote to Table 1.

they contained about 2.4 mg and 1.1 mg of full length hybrid for 1 litre of bacterial culture at $A_{600nm} = 2.5$. Their profiles in SDS-polyacrylamide gels were reproducible and therefore depended on the specific structure of the scFvs (Fig. 3).

We then submitted the preparations of Hyb19-22 and Hyb93-11 to an anion exchange chromatography for a further purification. We continuously monitored the absorbance of the column eluate at A_{280nm}, and also analysed individual fractions for their content in protein by electrophoresis through SDS-polyacrylamide gels and for their content in anti-$TrpB_2$ activity by indirect ELISA. We found that the full-length form of each hybrid was eluted in two distinct peaks of absorbance, by two different concentrations of NaCl, 50 mM and 100 mM. For Hyb19-22, the two peaks of absorbance were of equal importance. For Hyb93-11, the peak eluting at 100 mM was minor. The fractions that contained the full-length hybrid molecules were strongly active against $TrpB_2$. The fractions that did not contain any full-length hybrid were inactive. This anion exchange chromatography allowed us to purify the hybrids to >90% homogeneity (Fig. 3).

The scFvs of some antibodies can form dimers in which the V_L of one monomer associates with the V_H of the other monomer to reconstitute two active sites for the binding of the antigen (see Introduction). To test whether one of the purified fractions corresponded to a monomeric form of scFv-MalE and the other one to a dimeric form, we submitted them to a gel filtration chromatography. All the purified fractions contained a major protein species (>85%) whose Stokes radius was compatible with a monomer of scFv-MalE, and a minor species which could be a dimer.

Functional comparison between the scFv-MalE hybrids and the parental antibodies

We determined the dissociation constants at equilibrium in solution, K_D, for the complexes between $TrpB_2$ and either the parental antibodies or the purified scFv-MalE hybrids by competition ELISA at 4°C. We used variants Hyb19-22 and Hyb 93-11 for these experiments, as above. For mAb19 and Hyb19-22, we found K_D values of 0.13 nM and 0.19 nM, respectively. For mAb93 and Hyb93-11, we found 1.00 nM and 0.25 nM, respectively. Thus, the affinities of the hybrids were close to those of the corresponding native antibodies.

We used the BIAcore apparatus to monitor the kinetics of the interaction between $TrpB_2$, dissolved in the liquid phase, and either the parental antibodies or the scFv-MalE hybrids, immobilized on the sensor chip. We fitted single exponential functions to the association and dissociation profiles, derived the rate constants, k_{on} and k_{off}, and calculated the equilibrium dissociation constant, K_D', at the interface between the dissolved and immobilized molecules as described in the experimental section for the 'simple mechanism'. $K_D' = k_{off}/k_{on}$ is not an equilibrium dissociation constant in solution, but yet is useful to compare the binding of the same analyte to variants of a given ligand. We found that the values of k_{on}, k_{off} and K_D' for Hyb19-22 and Hyb93-11 were very similar to those of the parental antibodies (Table 3). The values of K_D' for the hybrids and for the parental antibodies (Table 3) were higher than the K_D ones (see above). The values of k_{off} and k_{on} for mAb19 and mAb93, measured in this work with the BIAcore apparatus (Table 3), were lower than those previously measured by spectrofluorimetry in solution (Friguet *et al*., 1989a, b; Larvor *et al*., 1991). The reasons for these differences have already been discussed (Azimzadeh and Van Regenmortel, 1990; Gruen *et al*., 1993).

We repeated the kinetic experiments using holo-$TrpB_2$ as the antigen (i.e. $TrpB_2$ with bound pyridoxal 5-phosphate) instead of apo-$TrpB_2$ (i.e. $TrpB_2$ without pyridoxal 5-phosphate). We found that mAb19 and Hyb19-22 on the one hand, mAb93 an Hyb93-11 on the other hand, bound holo-$TrpB_2$ with very similar kinetics. In particular, the ratios of the K_D's for the apo- and holo-forms of $TrpB_2$ were identical for the parental antibodies and for the hybrids (not shown). Thus, the parental antibodies and the hybrids had very similar properties of recognition towards both apo- and holo-$TrpB_2$.

Effects of the amino acid differences on the kinetics of binding

We compared the rates of interaction between $TrpB_2$ and the different variants of the scFv-MalE hybrids (Table 3). We found only small differences of k_{on} and k_{off} between the hybrids derived from mAb19. Their K_D' values were larger than the K_D' of the parental mAb19 by a factor 2.5 to 4.5. The situation was more contrasted for the hybrids derived from mAb93. Three of them, Hyb93-01, Hyb93-11 and Hyb93-29, had similar k_{on}s and k_{off}s and their K_D' values were about three times larger than the K_D' of mAb93. Two

Hyb19

```
GCGGCCCAGCCGGCCATGGCC CAGGT##A#CTGCAG#AGTC##GGGCTGAACTGCTGAAGCCTGGGGCTTCAGTG
 A  A  O  P  A  M  A / Q  V  #  L  Q  #  S  #  A  E  L  L  K  P  G  A  S  V
        << Signal    / 1   VH >>                          10
A#GTTGTCCTGCAAGGCTTCTGGCTACACCTTCACCAGCTCCTATTTGTACTGGGTGAAACAGAGGCCTGGACAA
 #  L  S  C  K  A  S  G  Y  T  F  T  S  S  Y  L  Y  W  V  K  Q  R  P  G  Q
   20                            30       CDR1                    40
GGCCTTGAGTGGATTGG#GAGATTAATCCTAGCAGTGGTGAAACTGACTTCAATGAGAAGTTCAAGACCAAGGCC
 G  L  E  W  I  G  E  I  N  P  S  S  G  E  T  D  F  N  E  K  F  K  T  K  A
                  50   52A          CDR2        60
ACACTGACTGTAGACAAATCCTCCAGTACAGCATTTTTGCAACTCAGCAGCCTGACATCTGAGGACTCTGCGGTC
 T  L  T  V  D  K  S  S  S  T  A  F  L  Q  L  S  S  L  T  S  E  D  S  A  V
      70                        80    82 -A -B -C 83
TATTACTGTAGTAGGTTCGATGTCTGGGG#CA#GG#ACCACGGTCA#CGTCTCCTCA #GTGGAGGCGGTTCA#GC
 Y  Y  C  S  R  F  D  V  W  G  Q  G  T  T  V  #  V  S  S / #  G  G  G  S  #
90          94-100 CDR3                      110  << VH  / 1     Linker
GGAGGTGGCTCTGGCGGT#G#GGATCG GACATCGAG#T#AC#CAGTCTCC##CCTCCCTATCTGCATCTGTGGGA
 G  G  G  S  G  G  #  G  S / D  I  E  #  T  Q  S  P  #  S  L  S  A  S  V  G
    Pentadecapeptide    15 / 1  VL >>                  10
GAAACTGTCACCATGACATGTCG#GCAAGTGAGAATATTTACAATAATTTAGCATGGTATCAGCAGAAACAGGGA
 E  T  V  T  M  T  C  R  A  S  E  N  I  Y  N  N  L  A  W  Y  Q  Q  K  Q  G
        20                    CDR1   30                      40
AAATCTCCTCAGCTCCTAGTCTATGGTACAGCAAGATTAGCAGATG#TATACCATCAAGGTTCAGTGGCAGTGGA
 K  S  P  Q  L  L  V  Y  G  T  A  R  L  A  D  #  I  P  S  R  F  S  G  S  G
                     50      CDR2                    60
TCAGGCACGCAGTTTTCTCTGAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAATTATTACTGTCAACATTTT
 S  G  T  Q  F  S  L  K  I  N  S  L  Q  P  E  D  F  G  N  Y  Y  C  Q  H  F
        70                        80                      90
CATGGTACTCCTCGGACGTTCGGTGGAGG#AC#AAG#TGGA##T#AAACGGGCG GCCGCTGGTACC AAAATC..
 H  G  T  P  R  T  F  G  G  G  T  K  L  E  #  K  R  A / A  A  G  T / K  I
     CDR3              100                  << VL  109/   Linker   / MalE >>
```

Hyb93

```
GCGGCCCAGCCGGCCATGGCC CAGGT##AGCTGCAG#AGTC#GG#GCTGAGCTGGTTAGGCCTGGGACTTCA
 A  A  Q  P  A  M  A / Q  V  #  L  Q  #  S  G  A  E  L  V  R  P  G  T  S
        << Signal    / 1   VH >>                          10
GTGAAGATGTCCTGCAAGGCTGCTGGATACACCTTCACTAACTACTACATAGGTTGGGTGAAGCAGAGGCCTGCA
 V  K  M  S  C  K  A  A  G  Y  T  F  T  N  Y  Y  I  G  W  V  K  Q  R  P  A
      20                          30       CDR1                    40
CATGGCCTTGAGTGGATTGGAGATATTTACCCTGGAGGTGGTTATCCTAACTACAATGAGAAGTTCA#GGGCAAG
 H  G  L  E  W  I  G  D  I  Y  P  G  G  G  Y  P  N  Y  N  E  K  F  #  G  K
                     50     52A           CDR2        60
GCCACACTGACTGCAGACACATCCTCCAGCACAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCC
 A  T  L  T  A  D  T  S  S  S  T  A  Y  M  Q  L  S  S  L  T  S  E  D  S  A
        70                        80    82 -A -B -C 83
ATCTATTACTGTGCAAGATCAGGGGTCTTTGACTATTGGGGCCAAGG#ACCACGGTCACCGTCTCCTCA GGT
 I  Y  Y  C  A  R  S  G  V  F  D  Y  W  G  Q  G  T  T  V  T  V  S  S / G
   90                96   100 CDR3                    110  << VH    / 1
GGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCG GACATCGAGCTCAC##A#TCTCC#GCATCT
 G  G  G  S  G  G  G  G  S  G  G  G  G  S / D  T  E  L  T  #  S  P  A  S
     Linker Pentadecapeptide          15/ 1   VL >>                10
TTGGCTGTGTCTCTAGGGCAGAAGGCCACCATCTCCTGCAAGGCCAGCAAAGAAGTCACTATATTTGGCTCTATA
 L  A  V  S  L  G  Q  K  A  T  I  S  C  K  A  S  K  E  V  T  I  F  G  S  I
                     20                26     CDR1          28     30
ATTGCTCTGCACTGGTACCAACAGAAACCAGGACAGCCACCCAAACTCATCTATAATGGAGCCAAACTAGAATCT
 I  A  L  H  W  Y  Q  Q  K  P  G  Q  P  P  K  L  I  Y  N  G  A  K  L  E  S
   CDR1                    40                46-48    50 CDR2
GGGGTCAGTGCCAGGTTCAGTGACAGTGGGTCTCAGAACCGCTCACCATTTGGAAATCAGCTCAACTTCACCCTC
 G  V  S  A  R  F  S  D  S  G  S  Q  N  R  S  P  F  G  N  Q  L  N  F  T  L
         60                  67  <<<<<< Extra Sequence >>>>>> 70
ACCATTGATCCTGTGGAGGCTGATGATGCAGCAACCTATTACTGTCTGCAAAATAAAGAGGTTCCGTACACGTTC
 T  I  D  P  V  E  A  D  D  A  A  T  Y  Y  C  L  Q  N  K  E  V  P  Y  T  F
                  80                90        CDR3
GGAGGGGG#AC#AAG#TGGAAAT#AAACGGGCG GCCGCTGGTACC AAAATC...
 G  G  G  T  K  L  E  I  K  R  A / A  A  G  T / K  I
  100               << VL   109/   Linker   /  MalE >>
```

Figure 2. Nucleotide sequences of the scFv-coding genes and deduced amino-acid sequences. The amino-acid residues are numbered according to Kabat *et al.* (1991). The positions at which the nucleotide or amino-acid residue varied between different clones, are indicated by "#", and the changes are listed in Tables 1 and 2. The nucleotide sequences of the CDRs are underlined. The sequences of the signal peptide and of the linker regions are italicized.

others, Hyb93-31 and Hyb93-34, had a higher K_{off} and a lower k_{on} than the other mAb93 hybrids, so that their K_D' values were 23 and 15 times higher, respectively, than the K_D' of mAb93. These two last hybrids gave weak signals during the initial screening of the bacterial clones by ELISA and their amylose-purified preparations contained large proportions of degraded molecules (Fig. 4). The amino acid sequences of Hyb93-31 and Hyb93-34 differed from the consensus sequence for mAb93 in one position and three positions, respectively, and thus only one change was common to both variants, the change of Gln6 into Glu in V_H (Table 2). Therefore, this amino acid change seems responsible for both reduced affinity and degradation of Hyb93-31 and Hyb93-34.

Discussion

Expression of functional scFv-MalE hybrids from plasmid pFBX

In a previous work, we have fused protein MalE with Fv or scFv fragments derived from D1.3, a monoclonal antibody directed against hen egg white lysozyme. We have shown that the resulting hybrid proteins could be purified by affinity chromatography on cross-linked amylose, as MalE, and that their affinities for lysozyme were close to that of the parental antibody (Brégégère *et al*., 1994; England *et al*., 1997). Here, we describe the plasmid vector pFBX, which enables one to clone scFv coding sequences and express them as scFv-MalE hybrids. The restriction sites *SfiI* and *NotI* of pFBX are compatible with those of other vectors,

Table 3. Rate and equilibrium constants for the parental antibodies and their scFv-MalE derivatives as measured with the BIAcore

Antibody	$\langle k_{off} \rangle$ (10^{-4}/s)	k_{on} (10^4/M/s)	K_D' (nM)
mAb19	0.88±0.04	3.52±0.26	2.5±0.3
Hyb19-04	1.08±0.13	1.27±0.06	8.5±1.4
Hyb19-12	1.55±0.04	1.50±0.23	10.3±1.8
Hyb19-20	1.27±0.11	1.21±0.16	10.5±2.3
Hyb19-22	1.22±0.06	1.97±0.32	6.2±1.3
Hyb19-24	1.40±0.13	1.21±0.08	11.6±1.9
Hyb19-27	1.16±0.07	1.33±0.14	8.7±1.4
mAb93	1.00±0.06	3.32±0.57	3.0±0.7
Hyb93-01	1.42±0.07	1.52±0.23	9.3±1.9
Hyb93-10		No protein	
Hyb93-11	1.46±0.11	1.78±0.38	8.2±2.9
Hyb93-29	1.50±0.06	1.70±0.20	8.8±1.4
Hyb93-31	4.0±0.6	0.59±0.01	68±11
Hyb93-34	3.8±3.5	0.86±0.03	44±6
mAb164	0.25±0.02	1.31±0.23	1.9±0.5

Kinetic profiles were recorded with the BIAcore apparatus for several concentrations of apo-$TrpB_2$, 40 nM≤C≤400 nM. Values of k_{off} and k_{obs} were obtained by fitting functions (1) and (2) to each of these profiles (simple model of the Experimental section). $\langle k_{off} \rangle$ is the mean value of the k_{off} parameter when C varies and it is given with its standard error. The value of k_{on} was obtained by fitting equation (3) to the experimental values of k_{obs} and C with the software Kaleidagraph. Its standard error in the fitting is indicated. $K_D' = \langle k_{off} \rangle / k_{on}$ is the equilibrium dissociation constant between the immobilized ligand (one of the scFv-MalE hybrids or monoclonal antibodies) and the soluble antigen (apo-$TrpB_2$). The relative error on K_D' was calculated by adding the relative errors on $\langle k_{off} \rangle$ and k_{on}.

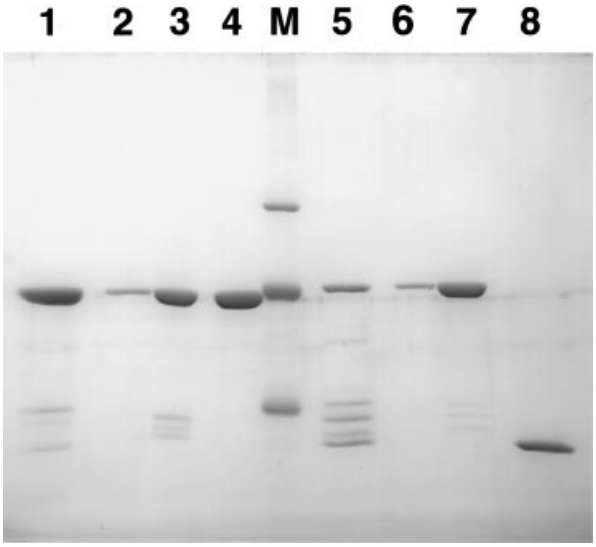


Figure 3. Gel analysis of the purified preparations of scFv-MalE hybrids. Hyb19-22 and Hyb93-11 were first purified by chromatography on a column of cross-linked amylose, then the partially purified preparations were further fractionated by chromatography on a MonoQ column. The fractions were analysed by electrophoresis through a 10% SDS-polyacrylamide gel and the proteins were stained with coomassie blue, Lanes 1–4, Hyb19-22; lanes 5–8, Hyb93-11; M, molecular mass markers (45, 66 and 97 kDa). Lanes 1 and 5, fractions eluted from the cross-linked amylose column; other lanes, fractions eluted from the MonoQ column. Lanes 3 and 7, fractions eluting at 50 mM NaCl; lanes 2 and 6, identical to lanes 3 and 7 respectively, except that there is 10 times less material; lane 4, fraction eluting at 100 mM NaCl; lane 8, fraction eluting at 75 mM NaCl and containing the main impurity seen in lane 5.

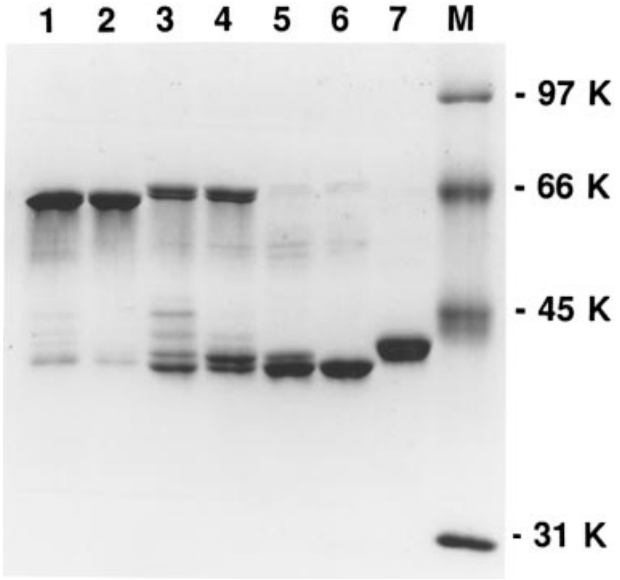


Figure 4. Degradation profiles of scFv-MalE hybrids. The hybrids were purified by chromatography on a column of cross-linked amylose and the purified preparations were analysed by electrophoresis through a 12% SDS-polyacrylamide gel. Lanes 1 and 2, 18 month and two-day-old samples of Hyb19-22; lanes 3 and 4, 18 month and two-day-old samples of Hyb93-11; lane 5, Hyb93-31; lane 6, Hyb93-34; lane 7, $TrpB_2$; M, molecular mass markers.

like pCANTAB6 (Pharmacia-Biotech), that are also used for the cloning and expression of scFv coding sequences (Hoogenboom *et al*., 1992). Using pFBX, we have cloned the variable genes of two monoclonal antibodies, mAb19 and mAb93, specific for different epitopes of protein $TrpB_2$. The corresponding scFv-MalE hybrids bound $TrpB_2$ specifically, with equilibrium and rate constants similar to those of the parental antibodies. We conclude that MalE is a valid vector for scFv fragments of diverse specificities.

Production, purification and stability of the scFv-MalE hybrids

The scFv-MalE hybrids derived from mAb19 and mAb93, were produced at low, non-toxic levels in strain HB2200. This weak expression was probably due to a read-through transcription of the hybrid gene from promoter *lacp*, which is located upstream from *malEp* in pFBX. It was sufficient to detect some anti-$TrpB_2$ activity in periplasmic extracts. The hybrids were optimally produced in strain PD28, under the control of promoter *malEp*, even though their production was slightly toxic. They were purified by affinity chromatography on cross-linked amylose. For variants Hyb19-22 and Hyb93-11, the yields of full-length molecules were about 1.0 and 0.5 mg, respectively, per litre of culture and per $A_{600\,nm}$ unit in Erlenmeyer flasks. These yields of seFv-MalE hybrids compare well with those of free scFvs, about 0.3 mg, obtained with other vectors (Skerra *et al*., 1991).

The partially purified preparations of hybrids contained incomplete molecules whose amount and pattern were specific of each antibody (Fig. 4). These incomplete molecules were due neither to a premature arrest of the transcription or translation of the hybrid genes nor to a degradation of the MalE portion of the hybrid proteins, because a deletion of the 30 C-terminal residues of MalE would abolish their binding to amylose (Duplay *et al*., 1987). Hence, they arose from a degradation of the scFv portion of the hybrid proteins. Variants Hyb93-31 and Hyb93-34 were more degraded than the other hybrids, probably due to the change of Gln6 into Glu in their V_H domains. This amino acid change could introduce the recognition sequence by a protease or destabilize the scFv portion of the hybrids. Hyb19-22 and Hyb93-11 displayed unchanged profiles in SDS-polyacrylamide gels (Fig. 4) and unchanged kinetics of interaction with $TrpB_2$ after storage during 18 months at 4°C in the presence of sodium azide. Thus, they were stable at 4°C *in vitro*. However, we observed that they became inactive after overnight incubation at 25°C under the conditions of competition ELISA. We could perform these experiments safely at 4°C.

We observed that variants Hyb19-22 and Hyb93-11 were eluted in two distinct peaks of absorbance from an anion exchange column, by 50 and 100 mM NaCl. The eluate fractions corresponding to these peaks were >90% pure and contained a strong anti-$TrpB_2$ activity. Analysis of the pure fractions by gel filtration chromatography showed that at least 85% of their content was monomeric scFv-MalE. Several hypotheses could explain this behaviour of the hybrids. We run the gel filtration chromatographies at higher NaCl concentration (150 mM) than the anion exchange or affinity chromatographies. scFv-MalE might form multimers at low salt concentration, in conditions where we know that the isolated protein MalE is monomeric (Blondel and Bedouelle, 1990). The monomers of scFv-MalE might adopt several conformations, for example by forming tertiary interactions between their scFv and MalE portions. Such tertiary interactions have been observed for other hybrids with MalE (Blondel *et al*., 1996). Whatever the explanation is, this phenomenon depended on the parental antibody, since it was important with mAb19, weak with mAb93 and non-existing with mAbD1.3 (Brégégère *et al*., 1994).

Unusual features of the mAb19 and mAb93 sequences

Because independent clones gave different levels of anti-$TrpB_2$ activity during the initial screening of the HB2200 transformants by ELISA, we determined the complete nucleotide sequences of six different cloned genes for each of antibodies mAb19 and mAb93. We could deduce consensus sequences for their rearranged variable genes from these independent sequences (Fig. 2, Tables 1 and 2). Both mAb19 and mAb93 belong to class γ1,k of mouse immunoglobulins G, according to their serological properties (L. Djavadi-Ohaniance, unpublished results). We compared the deduced amino acid sequences of the variable domains to the canonical sequences established by Kabat *et al*. (1991). The V_H sequence of mAb19 was very similar to the consensus sequence of subgroup II_B, with 94 identities over 117 positions. The V_L of mAb19 could belong to subgroups K_V, K_{IV} and K_{VI} with 70/108, 68/108 and 66/108 sequence identities, respectively. The V_H of mAb93 could belong to subgroups V_A, II_B and II_A with 88/115, 87/115 and 86/115 identities, respectively; the corresponding V_L displayed 82/105 identities with the consensus sequence of subgroup K_{III}, but two of its framework regions were not colinear with the canonical sequence (see below).

Both mAb19 and mAb93 had unusual features. The H-CDR3 region of mAb19 contained only 3 residues, which made it one of the shortest so far documented (Wu *et al*., 1993; Fig. 2). Since H-CDR3 is central in the binding site of antibodies, its shortening could be associated with a hollow shape, complementary to a protuberant epitope. The L-FR2 and L-FR3 regions of mAb93 did not align continuously with the canonical sequences: only one Leu residue was present instead of two at positions 46–47, and the decapeptide Gln–Asn–Arg–Ser–Pro–Phe–Gly–Asn–Gln–Leu replaced the dipeptide Gly–Thr at positions 68–69 (Fig. 2). These two features have been reported and discussed in an independent work on mAb93, which showed that none of them can be removed without impairing the binding of $TrpB_2$ (Ge *et al*., 1995).

Frequencies of changes in the scFv coding sequences

The 6 plasmid clones that we sequenced for each antibody, were all different. The nucleotide differences in the scFv coding sequences were limited, 6 ± 3 per coding sequence for mAb19 and 4 ± 2 for mAb93 on average when compared with the consensus sequences. These low figures indicated that the changes were due to cloning artefacts. They

suggested that the high proportion of negative bacterial clones in the initial screening for anti-$TrpB_2$ activity could result from non-sense or inactivating mutations.

Most of the nucleotide differences (35/44) were clustered in the 5′ and 3′ ends of the variable genes and probably resulted from the use of mixed or degenerate primers during the PCR amplifications. Four differences were clustered in the sequence linking the V_H and V_L genes of mAb19 and probably came from the DNA fragment used to assemble them in a scFv gene. This DNA linker is obtained by PCR amplification of an established construction in the original publication (Clackson *et al*., 1991). The remaining five differences were scattered along the sequences coding for the variable domains and could be attributed to replication errors.

For mAb19, 94 codons of V_H and 91 codons of V_L did not overlap the PCR primers. Therefore, the four nucleotide differences that we found in the six sequenced clones after a total of 67 rounds of PCR gave an error rate per nucleotide and per cycle equal to $2*4/(94+91)*3*6*67=4.10^{-5}$. A similar calculation for the single difference found in mAb93 gave $2*1/(97+103)*3*6*67=8.10^{-6}$ error per nucleotide per cycle. These figures correspond to those reported by others for PCR reactions or are lower (Eckert and Kunkel, 1991).

Consequences of the sequence differences

The mixed, degenerate or consensus primers that are designed to anneal with the FR1 and FR4 regions of the rearranged variable genes and to clone these genes by PCR, code at each position for amino acids that are commonly found in natural antibodies, and therefore should not be detrimental to the properties of the scFv fragments. Froyen *et al*. (1995) have cloned two scFv-coding sequences from the same hybridoma by two different methods, one based on PCR and the other one based on a more traditional procedure of cDNA synthesis. These authors did not observe any functional difference between the two variants of the scFv, although their sequences carried 6 differences of amino acid residues. Here, we found that the change of V_H-Gln6 into V_H-Glu6 decreased the affinity of the scFv-MalE hybrids derived from mAb93 for $TrpB_2$ and increased their degradation, as shown by variants Hyb93-31 and Hyb93-34. In contrast, this change of residue did not affect the properties of the scFv-MalE hybrids derived from mAb19, as shown by Hyb19-20. Ge *et al*. (1995) have cloned one Fab-coding sequence and one scFv-coding sequence from the hybridoma of mAb93 by two different methods, both based on PCR. In both cases the same antibody sequence was obtained. This sequence differs from the consensus sequence described here at the V_H positions Glu1, Val5, Glu6 and Leu9, and at the V_L positions Ile2 and Ile4. All these differences of sequence can be explained by differences in the PCR primers. These authors have reported a K_D value for their isolated scFv (5.3 nM; unlinked to MalE and containing V_H-Glu6) which is close to the K_D' values for variants Hyb93-01, Hyb93-10 and Hyb93-29 (8.2 to 9.3 nM; containing V_H-Gln6) and they have not mentioned any instability. Thus, the high K_D' values (44 and 68 nM) and the instability of Hyb93-31 and Hyb93-34 were not due to deleterious associations between the V_H-Glu6 residue and either the MalE domain or the unusual features of mAb93 since they were not observed for Hyb19-20 and for the isolated scFv of mAb93. The change of V_H-Gln6 into V_H-Glu6 may perturb the structural conformation of an scFv if it is not compensated by other changes, like those present in the variant of Ge *et al*. (1995). Therefore, one should check that the sequences brought by the primers, do not affect the activity or the stability of the final gene product when cloning rearranged variable genes by PCR.

Several improvements that increase the fidelity of cloning have been proposed. The use of DNA-polymerases that have an editing activity, can decrease the rate of errors during the numerous cycles of amplification. The use of primers that anneal to the leader sequences and the constant regions (Larrick *et al*., 1989; Jones and Bendig, 1991) rather than to the FR1 and FR4 regions of the rearranged variable genes, avoids introduction of changes in the scFv sequences by the PCR primers. The use of cloning methods that rely on PCR but not on the sequences of the variable genes, like the RACE method (rapid amplification of cDNA ends; Frohman *et al*., 1988; Ruberti *et al*., 1994), inverse PCR (Zwickl *et al*., 1990) and anchored PCR (Loh *et al*., 1989; Ratech *et al*., 1992; Heinrichs *et al*., 1995) also avoid these changes. According to our results, the use of these more complex methods is unnecessary from a functional point of view, and it is sufficient to compare the activities of periplasmic extracts towards the antigen in indirect ELISA, to find clones that express recombinant scFvs as active as the parental antibody.

Comparison of the kinetic models and data

Up to this point, we have reported an analysis of our kinetic data with the exponential functions (1) and (2), which correspond to a simple mechanism of interaction. This approximation was sufficient to compare the native antibodies with their hybrid counterparts. However, three

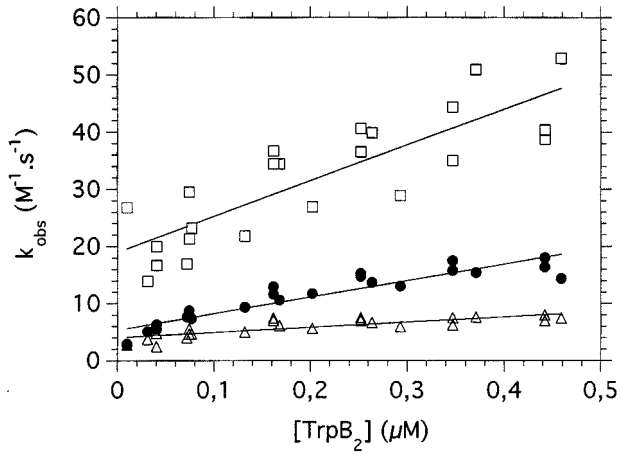


Figure 5. Variations of the apparent rate constants for antibody mAb19 with the molarity of $TrpB_2$. The values of k_{obs}, k_{obs1} and k_{obs2} were plotted *versus* the molarities of apo-$TrpB_2$, and function (3) was fitted to these experimental points to determine the association rate constants k_{on}, k_{on1} and k_{on2}. The coefficient of correlation R was equal to 0.92 for k_{obs} (●), 0.81 for k_{obs1} (△) and 0.84 for k_{obs2} (□) when all the points were considered, and to 0.95, 0.76 and 0.84 respectively when they were limited to 40 nM≤[$TrpB_2$]≤400 nM.

Table 4. Comparison of the single and dual models of interaction

	mAb19	Hyb19-22	mAb93	Hyb93-11	mAb164
Simple dissociation model					
$\langle k_{off} \rangle (10^{-4}/s)$	0.88 ± 0.04	1.22 ± 0.06	1.00 ± 0.06	1.63 ± 0.15	0.25 ± 0.02
$\langle \chi^2 \rangle$	1.3 ± 0.2	3.6 ± 0.5	4.4 ± 0.6	3.6 ± 0.9	1.4 ± 0.3
Simple association model					
$k_{on}*(10^4/M/s)$	3.52 ± 0.26	1.97 ± 0.32	3.32 ± 0.57	1.60 ± 0.42	1.31 ± 0.23
$\langle \chi^2 \rangle$	642 ± 122	349 ± 108	595 ± 162	53 ± 22	10 ± 6
Dual association model					
k_{on1} (10^4/M/s)	1.09 ± 0.19	0.51 ± 0.16	1.23 ± 0.39	0.86 ± 0.13	1.41 ± 0.49
k_{on2} (10^4/M/s)	7.2 ± 1.1	4.6 ± 1.1	11.1 ± 1.4	3.0 ± 1.5	7.4 ± 7.4
$\langle R_{eq2}/(R_{eq1}+R_{eq2}) \rangle$	0.32 ± 0.03	0.24 ± 0.03	0.33 ± 0.03	0.20 ± 0.03	0.08 ± 0.03
$\langle \chi^2 \rangle$	3.3 ± 0.2	2.3 ± 0.4	3.2 ± 1.1	0.9 ± 0.2	1.2 ± 0.3

Kinetics profiles were recorded with the BIAcore apparatus for a series of apo-$TrpB_2$ concentrations, 40 nM ≤ C ≤ 400 nM. The immobilized ligand is indicated on the top line. Exponential functions were fitted to the kinetic profiles as described in the Experimental section. A χ^2 statistical indicator was computed for each fitting. The values of k_{on}, k_{on1} and k_{on2} were determined by fitting function (3) to the experimental values of k_{obs}, k_{obs1}, k_{obs2} and C with the software Kaleidagraph. Their standard errors in the fitting are indicated. The fitting of function (5) to the association profiles yielded values of Req_1 and Req_2, the partial signal increases at steady state. To quantify the proportion of binding related to k_{obs2} (and therefore to k_{on2}), we calculated the expression $Req_2/(Req_1 + Req_2)$. The table gives the mean values $\langle k_{off} \rangle$, $\langle Req_2/(Req_1 + Req_2) \rangle$ and $\langle \chi^2 \rangle$, and their associated standard errors when C varied.

observations suggested to us that the interactions of mAb19, mAb93 and their hybrid derivatives with $TrpB_2$ followed more complex mechanisms. The apparent association rate constant k_{obs} for the simple mechanism varied linearly with the concentration C of $TrpB_2$ only between 40 and 400 nM (Fig. 5). The χ^2 parameter decreased up to 200 times when function (6), which is a sum of two exponential terms, was fitted to the association data instead of function (2) (Table 4). The χ^2 values associated with functions (2) and (6) increased with the concentration of $TrpB_2$ (not shown). The existence of all these complications for both the parental antibodies and the scFv-MalE hybrids was consistent with a common mechanism of interaction with $TrpB_2$.

Edwards *et al*. (1995) have also reported examples for which a sum of two exponential terms fitted better the kinetic profiles of interaction between the soluble analytes and the immobilized ligands than only one term. Their data excluded successive steps of binding and suggested an heterogeneity of the ligand molecules, either intrinsic or resulting from their immobilization. Foote and Milstein (1994) have observed that three out of 40 mouse monoclonal antibodies display complex kinetics of interaction with haptens in solution, and they have deduced the existence of conformational isomerisms in antibodies from this observation. They have proposed that this phenomenon is general, although often undetected.

In an attempt to determine which mechanism led to complex kinetics in our experiments with mAb19 and mAb93, we characterized the kinetics of interaction between mAb164, a third monoclonal antibody, and $TrpB_2$, its antigen. The epitope of mAb164 is included within residues 273–283 of TrpB (Friguet *et al*., 1989a). We found that function [2] fitted the association profile much better for mAb164 than for mAb19 and mAb93. The corresponding χ^2 value was relatively low for mAb164 (Table 4), and it increased only slightly with the concentration of $TrpB_2$ (not shown). This comparison of the three antibodies showed that mAb164 had a simpler mechanism of interaction with $TrpB_2$ than mAb19 and mAb93. Thus, the complex kinetics observed for mAb19 and mAb93 were not totally due to systematic technical causes, for example to an heterogeneous immobilization of the antibody molecule on the chip. The results rather suggested that the molecules of mAb19, mAb93 and their derivatives had an intrinsic conformational heterogeneity, which could pre-exist in the isolated scFv, as suggested by the data of Foote and Milstein (1994).

Conclusion

On the basis of this work and of a previous one (Brégégère *et al*., 1994), we can draw the following conclusions. Plasmid pFBX and the scFv-MalE hybrids constitute convenient tools to clone scFv-coding sequences, to produce scFvs in milligram amounts and to purify them independently of their antigen-binding properties. We found that the affinities of the scFv-MalE hybrids for the antigen, their rate constants of association with and dissociation from the antigen, and the deviations of the kinetic profiles from theoretical models, were similar to those observed with the parental antibodies. These results suggest that monoclonal antibodies and their scFv-MalE hybrids interact with their antigens by the same mechanisms, and can replace each other as conformational probes. The hybrids may be advantageous because they are produced in bacteria directly as monovalent reagents, can be purified readily, and can be modified by site-directed mutagenesis for a detailed analysis of the interaction with the antigen or for the introduction of fluorescent probes.

Acknowledgements

We thank Dr Malcolm Buckle for his expert and kind counselling in the use of the BIAcore apparatus.

References

Azimzadeh, A. and Van Regenmortel, M. H. (1990). Antibody affinity measurements. *J. Mol. Recogn.* **3**, 108–116.

Bedouelle, H. and Duplay, P. (1988). Production in *Escherichia coli* and one-step purification of bifunctional hybrid proteins which bind maltose—Export of the Klenow polymerase into the periplasmic space. *Eur. J. Biochem.* **171**, 541–549.

Better, M., Chang, C. P., Robinson, R. R. and Horwitz, A. H. (1988). *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* **240**, 1041–1043.

Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S. M., Lee, T., Pope, S. H., Riordan, G. S. and Whitlow, M. (1989). Single-chain antigen-binding proteins. *Science* **242**, 423–426.

Blondel, A. and Bedouelle, H. (1990). Export and purification of a cytoplasmic dimeric protein by fusion to the maltose-binding protein of *Escherichia coli. Eur. J. Biochem.* **193**, 325–330.

Blondel, A., Nageotte, R. and Bedouelle, H. (1996). Destabilizing interactions between the partners of a bifunctional fusion protein. *Prot. Engng* **9**, 231–238.

Brégégère, F., Schwartz, J. and Bedouelle, H. (1994). Bifunctional hybrids between the variable domains of an immunoglobulin and the maltose-binding protein of *Escherichia coli*: production, purification and antigen binding. *Protein Engng* **7**, 271–280.

Chaffotte, A. F. and Goldberg, M. E. (1987). Kinetics of the spontaneous transient unfolding of a native protein studied with monoclonal antibodies—monomer/dimer transition in the tryptophan-synthase β_2 subunit. *J. Mol. Biol.* **197**, 131–140.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D. and Winter, G. (1991). Making antibody fragments using phage display libraries. *Nature* **352**, 624–628.

Dattamajumdar, A. K., Jacobson, D. P., Hood, L. E. and Osman, G. E. (1996). Rapid cloning of any rearranged mouse immunoglobulin variable genes. *Immunogenetics* **43**, 141–151.

Desplancq, D., King, D. J., Lawson, A. D. and Mountain, A. (1994). Multimerization behaviour of single chain Fv variants for the tumor-binding antibody B72.3. *Prot. Engng* **7**, 1027–1033.

Duplay, P., Szmelcmann, S., Bedouelle, H. and Hofnung, M. (1987). Silent and functional changes in the periplasmic maltose-binding protein of *Escherichia coli* K12 - I. Transport of maltose *J. Mol. Biol.* **194**, 663–673.

Eckert, K. A. and Kunkel, T. A. (1991). DNA polymerase fidelity and the polymerase chain reaction. *PCR Methods and Applications* **1**, 17–24.

Edwards, P. R., Gill, A., Pollard-Knight, D. V., Hoare, M., Buckle, P. E., Lowe, P. A. and Leatherbarrow, R. J. (1995). Kinetics of protein-protein interactions at the surface of an optical biosensor. *Anal. Biochem.* **231**, 210–217.

England, P., Brégégère, F. and Bedouelle, H. (1997). Energetic and kinetic contributions of contact residues of antibody D1.3 in the interaction with lysozyme. *Biochemistry* **36**, 164–172.

Foote, J. and Milstein, C. (1994). Conformational isomerism and the diversity of antibodies. *Proc. Nat. Acad. Sci. USA* **91**, 10370–10374.

Friguet, B., Djavadi-Ohaniance, L. and Goldberg, M. E. (1989a). Polypeptide-antibody binding mechanism: conformational adaptation investigated by equilibrium and kinetic analysis. *Res. Immunol.* **140**, 355–376.

Friguet, B., Djavadi-Ohaniance, L. and Goldbert, M. E. (1989b). Immunochemical analysis of protein conformation. In *Protein Structure—A Practical Approach.* ed. by T. E. Creighton, pp. 287–310. IRL Press, Oxford.

Frohman, M. A., Dush, M. K. and Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**, 8998–9002.

Froyen, G., Hendrix, D., Ronsse, I., Fiten, P., Martens, E. and Billiau, A. (1995). Effect of VH and VL consensus-specific primers on the binding and neutralizing potential of a single-chain Fv directed towards HuIFN-g. *Molec. Immunol.* **32**, 515–521.

Ge, L., Lupas, A., Peraldi-Roux, S., Spada, S. and Plückthun, A. (1995). A mouse Ig-kappa domain of very unusual framework structure loses function when converted to the consensus. *J. Biol. Chem.* **270**, 12446–12451.

Goldberg, M. E. (1991). Investigating protein conformation, dynamics and folding with monoclonal antibodies. *T.I.B.S.* **16**, 358–362.

Gopinathan, K. P., Weymouth, L. A., Kunkel, T. A. and Loeb, L. A. (1979). Mutagenesis in vitro by DNA polymerase from an RNA tumour virus. *Nature* **278**, 857–859.

Griffiths, A. D., Malmqvist, M., Marks, J. D., Bye, J. M., Embleton, M. J., McCafferty, J., Baier, M., Holliger, K. P., Gorick, B. D., Hughes-Jones, N. C., Hoogenboom, H. R. and Winter, G. (1993). Human anti-self antibodies with high specificity from phage display libraries. *EMBO J.* **12**, 725–734.

Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., Allison, T. J., Prospero, T. D., Hoogenboom, H. R., Nissim, A., Cox, J. P. L., Harrison, J. L., Zaccolo, M., Gherardi, E. and Winter, G. (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J.* **13**, 3245–3260.

Gruen, L. C., Kortt, A. A. and Nice, E. (1993). Determination of relative binding affinity of influenza virus N9 sialidases with the Fab fragment of monoclonal antibody NC41 using biosensor technology. *Eur. J. Biochem.* **217**, 319–325.

Heinrichs, A., Milstein, C. and Gherardi, E. (1995). Universal cloning and direct sequencing of rearranged antibody V genes using C region primers, biotin-capture cDNA and one-side PCR. *J. Immunol. Meth.* **178**, 241–251.

Högberg-Raibaud, A. and Goldberg, M. E. (1977). Isolation and characterization of independently folding regions of the beta chain of *Escherichia coli* tryptophan synthetase. *Biochemistry* **16**, 4014–4020.

Hoogenboom, H. R., Marks, J. D., Griffiths, A. D. and Winter, G. (1992). Building antibodies from their genes. *Immunol. Rev.* **130**, 41–68.

Huse, W. D., Sastry, L., Iverson, S. A., Kang, A. S., Alting-Mees, M., Burton, D. R., Benkovic, S. J. and Lerner, R. A. (1989). Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* **246**, 1275–1281.

Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R. and Oppermann, H. (1988). Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **85**, 5879–5883.

Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W. and Davies, D. R. (1988). Three-dimensional structure of the tryptophan synthase $a_2\beta_2$ multienzyme complex from *S. typhimurium. J. Biol. Chem.* **263**, 17857–17871.

Jones, S. T. and Bendig, M. M. (1991). Rapid PCR-cloning of full-length mouse immunoglobulin variable regions. *Bio/Technology* **9**, 88–89.

Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. and Foeller, C. (1991). *Sequences of proteins of immunological interest*, 5th edition, National Institutes of Health publication, Bethesda, pp. 151–270 and 339–511.

Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367–382.

Larrick, J. W., Danielsson, L., Brenner, C. A., Abrahamson, M., Fry, K. E. and Borrebaeck, C. A. (1989). Rapid cloning of rearranged immunoglobulin genes from human hybridoma cells using mixed primers and the polymerase chain

reaction. *Biochem. Biophys. Res. Com.* **160**, 1250–1256.
Larvor, M. P., Djavadi-Ohaniance, L., Nall, B. and Goldberg, M. E. (1994). Measurement of the dissociation rate constant of antigen/antibody complexes in solution by enzyme-linked immunosorbent assay. *J. Immunol. Meth.* **170**, 167–175.
Loh, E. Y., Elliott, J. F., Cwirla, S., Lanier, L. L. and Davis, M. M. (1989). Polymerase chain reaction with single-sided specificity: analysis of T cell receptor delta chain. *Science* **243**, 217–220.
Mallender, W. D., Carrero, J. and Voss, E. W. Jr. (1996). Comparative properties of the single chain antibody and Fv derivatives of mAb 4-4-20. Relationship between interdomain interactions and the high affinity for fluorescein ligand. *J. Biol. Chem.* **271**, 5338–5346.
Mead, D. A., Szczena-Skorupa, E. and Kemper, B. (1986). Single-stranded DNA 'blue' T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. *Protein Engng* **1**, 67–74.
Miles, E. W. (1970). The B protein of Escherichia coli tryptophan synthetase. I. Effects of sulfhydryl modification on enzymatic activities and subunit interactions. *J. Biol. Chem.* **245**, 6016–6025.
Navon, A., Schulze, A. J., Guillou, Y., Zylinski, C. A., Baleux, F., Expert-Bezançon, N., Friguet, B., Djavadi-Ohaniance, L. and Goldberg, M. E. (1995). Importance of residues 2-9 in the immunoreactivity, subunit interactions, and activity of the β_2 subunit of *Escherichia coli* tryptophan synthase. *J. Biol. Chem.* **270**, 4255–4261.
Nicholls, P. J., Johnson, V. G., Blanford, M. D. and Andrew, S. M. (1993). An improved method for generating single-chain antibodies from hybridomas. *J. Immunol. Meth.* **165**, 81–91.
Onoue, K., Yagi, Y., Grossberg, A. L. and Pressman, D. (1965). Number of binding sites of rabbit macroglobulin antibody and its subunits. *Immunochem.* **2**, 401–415.
Orlandi, R., Gussow, D. H., Jones, P. T. and Winter, G. (1989). Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86**, 3833–3837.
O'Shannessy, D. J., Brigham-Burke, M., Soneson, K. K., Hensley, P. and Brooks, I. (1993). Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: use of nonlinear least squares analysis methods. *Anal. Biochem.* **212**, 457–468.
Ratech, H. (1992). Rapid cloning of rearranged immunoglobulin heavy chain genes from human B-cell lines using anchored polymerase chain reaction. *Biochem. Biophys. Res. Comm.* **182**, 1260–1263.
Richarme, G. (1982). Associative properties of the Escherichia coli galactose binding protein and maltose binding protein. *Biochem. Biophys. Res. Commun.* **105**, 476–481.
Ruberti, F., Cattaneo, A. and Bradbury, A. (1994). The use of the RACE method to clone hybridoma cDNA when V region primers fail. *J. Immunol. Meth.* **173**, 33–39.
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). In *Molecular Cloning, a Laboratory Manual*, pp. 125–128. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
Schier, R., Marks, J. D., Wolf, E. J., Apell, G., Wong, C., McCartney, J. E., Bookman, M. A., Huston, J. S., Houston, L. L., Weiner, L. M. and Adams, G. P. (1995). *In vitro* and *in vivo* characterization of a human anti-c-erbB-2 single chain Fv isolated from a filamentous phage antibody library. *Immunotechnology* **1**, 73–81.
Skerra, A. and Plückthun, A. (1988). Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli. Science* **240**, 1038–1041.
Skerra, A., Pfitzinger, I. and 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.
Ward, E. S., Güssow, D., Griffiths, A. D., Jones, P. T. and Winter, G. (1989). Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli. Nature* **341**, 544–546.
Wilson-Miles, E. (1991). Structural basis for the catalysis by tryptophan synthase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **64**, 93–172.
Whitlow, M., Bell, B. A., Feng, S. L., Filpula, D., Hardman, K. D., Hubert, S. L., Rollence, M. L., Wood, J. F., Schott, M. E., Milenic, D. E., Yokota, T. and Schlom, J. (1993). An improved linker for single-chain Fv with reduced aggregation and enhanced proteolytic stability. *Prot. Engng* **6**, 989–995.
Whitlow, M., Filpula, D., Rollence, M. L., Feng, S. L. and Wood, J. F. (1994). Multivalent Fvs: characterization of single-chain Fv oligomers and preparation of a bispecific Fv. *Prot. Engng* **7**, 1017–1026.
Wu, T. T., Johnson, G. and Kabat, E. A. (1993). Length distribution of CDRH3 in antibodies. *PROTEINS: Structure, Function and Genetics* **16**, 1–7.
Zetina, C. R. and Goldberg, M. E. (1982). Kinetics of renaturation and self-assembly of intermediates on the pathway of folding of the beta 2-subunit of Escherichia coli tryptophan-synthetase. *J. Mol. Biol.* **157**, 133–148.
Zhou, H., Fisher, R. J. and Papas, T. S. (1994). Optimization of primer sequences for mouse scFv repertoire display library construction. *Nucl. Acids Res.* **22**, 888–889.
Zwickl, M., Zaninetta, D., McMaster, G. K. and Hardman, N. (1990). Selective cloning of B cell hybridoma-specific rearranged immunoglobulin gene loci using the polymerase chain reaction. *J. Immunol. Meth.* **130**, 49–55.

