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

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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\textsubscript{2} subunit of \textit{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 \textit{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\textsuperscript{TM} biosensor apparatus were used to compare the energetics and kinetics with which the parental antibodies and the hybrids bound TrpB\textsubscript{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\textsubscript{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.


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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 \textit{et al.}, 1989; Orlandi \textit{et al.}, Clackson \textit{et al.}, 1991), express them in \textit{Escherichia coli} (Better \textit{et al.}, 1988; Skerra and Plückthun, 1988), and purify them independently of their antigen binding properties (Brégégère \textit{et al.}, 1994; Griffiths \textit{et al.}, 1994; Schier \textit{et al.}, 1995). Because the association between the variable domains, V\textsubscript{H} and V\textsubscript{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 \textit{et al.}, 1989; Huston \textit{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 \textit{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 \textit{E. coli}, as conformational probes, concerns the identity of their antigen-recognition properties. The S’ 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
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 \( V_\text{H} \) and \( V_\text{L} \) domains in an scFv fragment and the absence of a C\( _\text{H} \) domain can perturb the conformation of the antigen-binding site (Whitlow et al., 1993; Desplanq et al., 1994; Mallender et al., 1996). The scFv molecules can sometimes form dimers and multimers (Griffiths et al., 1993; Desplanq 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., 1994). 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\textsuperscript{TM} apparatus, based on surface plasmon resonance, to compare the energetics and kinetics with which the parental antibodies and the different variants of the hybrids bind TrpB\( _2 \).

When combined with previous studies (Brégégère et al., 1991; 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, \( \Delta \text{malE444, malT}^- \)) (Duplay et al., 1987), plasmids pVD10 (Brégégère et al., 1994) and pTZ18R (Mead et al., 1986) have been described. Promoter \( \text{malEp} \) is fully silent in HB2200 and constitutive in PD28. The derivatives of HB2200 and PD28 that contained plasmids, were grown with LB or 2\( \times \)YT medium (Difco) in the presence of 100 \( \mu \)g/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 \( \text{malEp} \), 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-Biotec (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 between the scFv fragment of monoclonal antibody D1.3 and protein MalE, under the control of promoter \( \text{malEp} \) (Brégégère et al., 1994). Insertion of this fragment into pTZ18R puts \( \text{malEp} \) in tandem with the promoter lacP. We generated SfiI, Ncol, 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 \( \text{malEp} \), close to its 3'-end, and the NotI site was introduced at the boundary between the \( V_\text{L} \) 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-Biotec). 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 \( V_\text{L} \) 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 \( V_\text{H} \) and \( V_\text{L} \), 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°C–55°C–72°C for mAb19; 94°C–51°C–72°C for mAb93). The \( V_\text{H} \) and \( V_\text{L} \) coding sequences were linked with a central DNA segment, coding for (Gly\text{Ser})\textsubscript{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 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, 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, of holo-TrpB, 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.

**Measurements of the equilibrium and rate constants**

We determined the equilibrium dissociation constants ($K_D$) between TrpB 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 ml/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 molecules because TrpB is formed by the non-covalent association of two subunits. The covalent coupling of TrpB 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 would then be definitively lost. We used the dissociation constants of TrpB, either into monomers, in the absence and in the presence of pyridoxal 5-phosphate, to calculate the actual molarities of the TrpB dimers in the BIAcore experiments (Chaffotte and Goldberg, 1987; Wilson-Miles, 1991). These calculated

Figure 1. pFBX, a phagemid for the expression of scFv-MalE hybrids. **Top:** partial restriction map of pFBX. The boxes represent open reading frames. **Bottom:** sequence alignments of pVD10, pFBX and pCANTAB5 around the cloning sites. The SfiI (GGCCCAGCCGGCC) and NotI (GCGGCCGC) sites, that are used for the insertion of the scFv-coding fragments, are underlined. The sites for NcoI (CCATGG) and for KpnI (GGTACC) are in italics.
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 $\chi^2$ parameter. We first fitted the simple exponential function:

$$ R = R_0 e^{-k_{on} t - k_{off} t} $$

(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, $R_0$ and $t_0$ are the values of $R$ and $t$ at the start of the measures, and $k_{on}$ and $k_{off}$ are fitting parameters.

Similarly, we fitted the function:

$$ R = k_0 R_0 / k_{obs} + (1 - e^{-k_{on} t - k_{off} t}) + R_d $$

(2)

to the profiles of association of TrpB$_2$ to the ligand, where $t_d$ is the start time of the association, and $R_0$, $k_0$ 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, $k_{on}$ is the association 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:

$$ k_{obs} = k_{on} C + k_{off} $$

(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

$$ K_D' = k_{off} / k_{on} $$

(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:

$$ R = R_1 e^{-k_{on1} t - k_{off1} t} + (R_0 - R_1) e^{-k_{on2} t - k_{off2} t} $$

(5)

$$ R = R_{eq1} (1 - e^{-k_{on1} t - k_{off1} t}) + R_{eq2} (1 - e^{-k_{on2} t - k_{off2} t}) + R_d $$

(6)

where $R$, $R_0$, $t$, $t_d$ and $t_0$ are like in (1) and (2), and $k_{on1}$, $k_{off1}$, $k_{on2}$, $k_{off2}$, $R_1$, $R_2$, $R_{eq1}$, and $R_{eq2}$ 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, $k_{on1}$ and $k_{off1}$ on the one hand, and $k_{on2}$ and $k_{off2}$ on the other hand represent partial rate constants and are linked by linear relationships similar to (3). $R_1$ is the portion of $R_0$ that is attributable to one of the complexes, $R_{eq1}$ and $R_{eq2}$ 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\textsubscript{H} and V\textsubscript{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\textsubscript{H} and one at the junction between the CDR2 and FR3 regions of V\textsubscript{L} in the case of mAb19; and one in the CDR2 of V\textsubscript{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

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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\textsubscript{L} and V\textsubscript{H} 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.
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 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 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_{H} of one monomer associates with the V_{L} 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}'s, at the interface between the dissolved and immobilized molecules as described in the experimental section for the 'simple mechanism'. K_{D}'s are 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}'s for Hyb19-22 and Hyb93-11 were very similar to those of the parental antibodies (Table 3). The values of K_{D}'s 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 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}.

### Table 2. Sequence variations in the clones derived from mAb93

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<th>93-34</th>
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See footnote to Table 1.
Effects of the amino acid differences on the kinetics of binding

We compared the rates of interaction between TrpB 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 $k_{on}$ and $k_{off}$s and their $K_D$ values were about three times larger than the $K_D$ of mAb93. Two

**Hyb19**

```
GCGGCCACCCGCGCCATGCCGAGGTCA#ACCTCAAGATCCG#GCGCTGACATGCTGACTGGCATGCTGGTCGCTAG
A A Q P A M A / Q V / I L Q / # S A E L L K P G A S V
<< Signal / 1 VH >>
```

**Hyb93**

```
GCGGCCACCCGCGCCATGCCGAGGTCA#ACCTCAAGATCCG#GCGCTGACATGCTGACTGGCATGCTGGTCGCTAG
A A Q P A M A / Q V / I L Q / # S A E L L K P G A S V
<< Signal / 1 VH >>
```

others, Hyb93-31 and Hyb93-34, had a higher $K_{\text{off}}$ and a lower $k_{\text{on}}$ 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

<table>
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<tr>
<th>Antibody</th>
<th>$k_{\text{on}}$ ($10^4$/s)</th>
<th>$k_{\text{off}}$ ($10^4$/M/s)</th>
<th>$K_D$ (nM)</th>
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<td>3.52±0.26</td>
<td>2.5±0.3</td>
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<td>Hyb19-04</td>
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<td>1.21±0.18</td>
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<td>1.97±0.32</td>
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<td>Hyb19-24</td>
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<td>Hyb93-29</td>
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<td>1.70±0.20</td>
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<tr>
<td>Hyb93-31</td>
<td>4.0±0.6</td>
<td>0.59±0.01</td>
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<tr>
<td>Hyb93-34</td>
<td>3.8±3.5</td>
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<td>44±6</td>
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<tr>
<td>mAb164</td>
<td>0.25±0.02</td>
<td>1.31±0.23</td>
<td>1.9±0.5</td>
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Kinetic profiles were recorded with the BIAcore apparatus for several concentrations of apo-TrpB$_2$, 40 nM ≤ $C$ ≤ 400 nM. Values of $k_{\text{off}}$ and $k_{\text{on}}$ were obtained by fitting functions (1) and (2) to each of these profiles (simple model of the Experimental section). ($k_{\text{on}}$) is the mean value of the $k_{\text{on}}$ parameter when $C$ varies and it is given with its standard error. The value of $k_{\text{on}}$ was obtained by fitting equation (3) to the experimental values of $k_{\text{obs}}$ and $C$ with the software Kaleidagraph. Its standard error in the fitting is indicated. $K_D$ = $k_{\text{off}}$/($k_{\text{on}}$) 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 $k_{\text{off}}$ and $k_{\text{on}}$.

![Figure 3](image1.png)

**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](image2.png)

**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.
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-TrpB2 activity in periplasmic extracts. The hybrids were optimally produced in strain PD28, under the conditions of competition ELISA. We contained a strong anti-TrpB2 activity. Analysis of the pure exchange column, by 50 and 100 mM NaCl. The eluate eluted in two distinct peaks of absorbance from an anion chromatography on cross-linked amylose. For variants at 25°C under the conditions of competition ELISA. We observed that variants Hyb19-22 and Hyb93-11 were more degraded than the other hybrids, 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 litre of periplasmic extracts. These yields of scFv-MalE hybrids compare well with those of free scFvs, about 0.3 mg, obtained with other vectors.

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 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 VH domains. This amino acid change could introduce one or two deletions of the scFv shape, complementary to a protuberant epitope. The L-FR2 domains to the canonical sequences established by Kabat et al. (1991). The VH sequence of mAb19 was very similar to the consensus sequence of subgroup IIb, with 94 identities over 117 positions. The VL of mAb19 could belong to subgroup IIb, with 70/108, 68/108 and 66/108 sequence identities, respectively. The VH of mAb93 could belong to subgroups IIIa, IIb and Ia, with 88/115, 87/115 and 86/115 identities, respectively; the corresponding VL displayed 82/105 identities with the consensus sequence of subgroup IIb, but two of its framework regions were not colinear with the canonical sequence (see below).

Both mAb19 and mAb93 had unusual features. The HCDR3 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 described in an independent work on mAb93, which showed that none of them can be removed without impairing the binding of TrpB2 (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-TrpB2 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<sub>H</sub> and V<sub>L</sub> 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<sub>H</sub> and 91 codons of V<sub>L</sub> did not overlap the PCR primers. Therefore, the four nucleotide differences that we found in the six sequenced clones after PCR amplification of an established construction in the original publication (Clackson et al., 1991) resulted from the use of mixed or degenerate primers 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.

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<sub>H</sub>-Gln6 into V<sub>H</sub>-Glu6 decreased the affinity of the scFv-MalE hybrids derived from mAb93 for TrpB2 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<sub>H</sub> positions Glu1, Val5, Glu6 and Leu9, and at the V<sub>L</sub> positions Ile2 and Ile4. All these differences of sequence can be explained by differences in the PCR primers. These authors have reported a K<sub>0</sub> value for their isolated scFv (5.3 nM; unlinked to MalE and containing V<sub>H</sub>-Glu6) which is close to the K<sub>0</sub>′ values for variants Hyb93-01, Hyb93-10 and Hyb93-29 (8.2 to 9.3 nM; containing V<sub>H</sub>-Gln6) and they have not mentioned any instability. Thus, the high K<sub>0</sub>′ values (44 and 68 nM) and the instability of Hyb93-31 and Hyb93-34 were not due to deleterious associations between the V<sub>H</sub>-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<sub>H</sub>-Glu6 into V<sub>H</sub>-Gln6 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
observations suggested to us that the interactions of mAb19, mAb93 and their hybrid derivatives with TrpB followed more complex mechanisms. The apparent association rate constant \( k_{on} \) for the simple mechanism varied linearly with the concentration \( C \) of TrpB, only between 40 and 400 nM (Fig. 5). The \( \chi^2 \) 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), which is a sum of two exponential terms, was fitted to the experimental data. Table 4 gives the mean values of \( k_{on} , k_{off} , k_{on1} , k_{on2} , k_{off1} , k_{off2} , C \), and their associated standard errors when \( C \) varied.

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

<table>
<thead>
<tr>
<th></th>
<th>mAb19</th>
<th>Hyb19-22</th>
<th>mAb93</th>
<th>Hyb93-11</th>
<th>mAb164</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{off} ) (10(^{-4})/s)</td>
<td>0.88±0.04</td>
<td>1.22±0.06</td>
<td>1.00±0.06</td>
<td>1.63±0.15</td>
<td>0.25±0.02</td>
</tr>
<tr>
<td>( \chi^2 )</td>
<td>1.3±0.2</td>
<td>3.6±0.5</td>
<td>4.4±0.6</td>
<td>3.6±0.9</td>
<td>1.4±0.3</td>
</tr>
</tbody>
</table>

Simple association model

\( k_{on} \) (10\(^{4}\)/M/s)

- mAb19: 3.52±0.26
- Hyb19-22: 1.97±0.32
- mAb93: 3.21±0.57
- Hyb93-11: 1.60±0.42
- mAb164: 1.31±0.23

\( \chi^2 \)

- mAb19: 642±122
- Hyb19-22: 349±108
- mAb93: 595±162
- Hyb93-11: 53±22
- mAb164: 10±6

Dual association model

\( k_{on} \) (10\(^{4}\)/M/s)

- mAb19: 1.09±0.19
- Hyb19-22: 0.51±0.16
- mAb93: 1.23±0.29
- Hyb93-11: 0.86±0.13
- mAb164: 1.41±0.49

\( R_{eq2} / (R_{eq1} + R_{eq2}) \)

- mAb19: 0.32±0.03
- Hyb19-22: 0.24±0.03
- mAb93: 0.33±0.03
- Hyb93-11: 0.20±0.03
- mAb164: 0.08±0.03

\( \chi^2 \)

- mAb19: 2.3±0.4
- Hyb19-22: 2.3±0.4
- mAb93: 3.2±1.1
- Hyb93-11: 0.9±0.2
- mAb164: 1.2±0.3

Kinetics profiles were recorded with the BIAcore apparatus for a series of apo-TrpB 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 \( \chi^2 \) statistical indicator was computed for each fitting. The values of \( k_{on} , k_{off} , k_{on1} , k_{on2} , k_{off1} , k_{off2} , C \) with the software Kaleidagraph. Their standard errors in the fitting are indicated. The fitting of function (5) to the association profiles yielded values of \( R_{eq2} \), and \( R_{eq1} \), the partial signal increases at steady state. To quantify the proportion of binding related to \( k_{on2} \) (and therefore to \( k_{off2} \)), we calculated the expression \( R_{eq2} / (R_{eq1} + R_{eq2}) \). The table gives the mean values of \( k_{on} \), \( k_{off} \), \( R_{eq2} / (R_{eq1} + R_{eq2}) \), and \( \chi^2 \), and their associated standard errors when \( C \) varied.

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.

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References


