Protein Engineering vol.7 no.2 pp.271–280, 1994

Bifunctional hybrids between the variable domains of an immunoglobulin and the maltose-binding protein of *Escherichia coli*: production, purification and antigen binding

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Hybrids were constructed between the maltose-binding protein of *Escherichia coli* (MalE) and the variable domains (V-domains) of D1.3, a mouse antibody directed against hen lysozyme. Each V-domain was fused with the C- or N-terminus of MalE and expressed in *E.coli*, either alone or associated with the other V-domain, as a heterodimer (Fv) or as a single-chain fragment (scFv). The hybrids were exported into the bacterial periplasm, purified by affinity chromatography on cross-linked amylose and separated from incomplete products by ion-exchange chromatography. Hybrids between MalE and Fv bound the antigen specifically, with affinities increased up to 10-fold when compared to native D1.3. This strongly suggests that MalE contributed to the binding. The affinities and specificities of the different hybrids, as well as their levels of contamination by incomplete products, depended on their fusion pattern with MalE. Hybrids between MalE and either single V-domain also bound hen lysozyme specifically, which shows that each V-domain can recognize the antigen when fused with MalE. The high affinity of V_H–MalE (K_D = 3 nM) could be due to both participation of MalE in the binding and a conformational adaptation of the lone V-domain.

Key words: antibody fragments/antigen binding/expression vectors/fusion proteins/immunoglobulin domains/maltose-binding protein

Introduction

Antibodies can be raised against most macromolecules. They specifically recognize and bind antigens through six short complementarity-determining regions (CDRs) located in their N-terminal variable domains (V-domains). The V-domains of the heavy and light chains can be cleaved from the constant domains to form a small, heterodimeric variable fragment or Fv (Givol, 1991). To improve the heterodimer stability, the V-domains can be connected together by a polypeptide linker, at the genetic level, to form a single-chain Fv or scFv (Glockshuber *et al.*, 1990; Johnson and Bird, 1991). Alternatively, they can be bound together by an interchain disulphide bridge or by chemical cross-linking (Plückthun, 1992). Such fragments bind antigens with affinities similar to those of the native antibodies.

The most common bacterial host for genetic engineering, *Escherichia coli*, cannot fold complete antibodies properly (Cabilly *et al.*, 1984), but it can produce Fv or scFv fragments in an active or renaturable form (Skerra and Plückthun, 1988; Field *et al.*, 1989). Several bacterial systems have been developed to produce antigen-binding fragments of antibodies (Winter and Milstein, 1991; Morrison, 1992; Plückthun, 1992). With the help of these systems, the interactions between antibodies and their antigens or between catalytic antibodies and their substrates were studied by site-directed mutagenesis (Baldwin and Schultz, 1989; Foote and Winter, 1992; Riechmann *et al.*, 1992).Pharmacological tools were constructed by linking scFvs with protein domains having various effector functions (Traunecker *et al.*, 1991; Haber, 1992). Libraries of antibody fragments were constructed in lambda phages (Huse *et al.*, 1989; Mullinax *et al.*, 1990) and in M13 phages (Clackson *et al.*, 1991; Hoogenboom *et al.*, 1992; Barbas *et al.*, 1993). In the latter case, the phages expose the antibody fragment on their surface and those coding for predefined binding specificities can be selected by immunoadsorption.

Because of the many potential applications of such antibody fragments, appropriate methods for their production and purification need to be developed. Affinity chromatography on an immobilized antigen is not always feasible, because the antigen may be rare or unstable and the engineered fragments may have altered binding properties. Moreover, elution from immunoadsorbents generally requires partial denaturation at low or high pH. A possible alternative consists of purifying antibody fragments as fusions with a protein that specifically binds an immobilized ligand. MalE, the maltose-binding protein of *E.coli*, has been used as a fusion partner to produce different kinds of proteins in an active form, to export them into the bacterial periplasm and to purify them by affinity chromatography on cross-linked amylose (Bedouelle and Duplay, 1988; Maina *et al.*, 1988; Blondel and Bedouelle, 1990; Szmelcmann *et al.*, 1990). We therefore expected MalE to be an appropriate vector for V-domains.

In this work, we describe the construction, production in *E.coli* and purification of hybrids between MalE and the V-domains of antibody D1.3 and we report the characterization of their antigen-binding properties. D1.3 is a mouse immunoglobulin with a γ_1 heavy chain and a κ light chain, directed against hen egg white lysozyme (HEL). We chose this particular antibody because the 3-D structures of the complexes formed by its Fab and Fv fragments with HEL have been determined by X-ray crystallography (Amit *et al.*, 1986; Bhat *et al.*, 1990). The capacity for purifying mutant V-domains in fusion with MalE, combined with the availability of structural data, should facilitate a rational study of the interactions between the D1.3 binding site and its antigen by site-directed mutagenesis.

Materials and methods

Phages, plasmids and bacterial strains

Derivatives of phage M13mp9, that carry cDNA inserts coding for the V-domains of antibody D1.3 (Amit *et al.*, 1986; Ward *et al.*, 1989b), were kindly provided by Dr Greg Winter. Plasmid pRE3, a derivative of pUC13 (Yanisch-Perron *et al.*, 1985), carries the *phoA* gene of *E.coli* (Chang *et al.*, 1986) on a *Hin*dIII–*Sal*I fragment and was provided by Dr Patricia Berg. Phage M13mp18(*4am*)*malE* (Martineau *et al.*, 1990), plasmids pPD1 (Duplay *et al.*, 1984) and pPD140 (Duplay *et al.*, 1987),

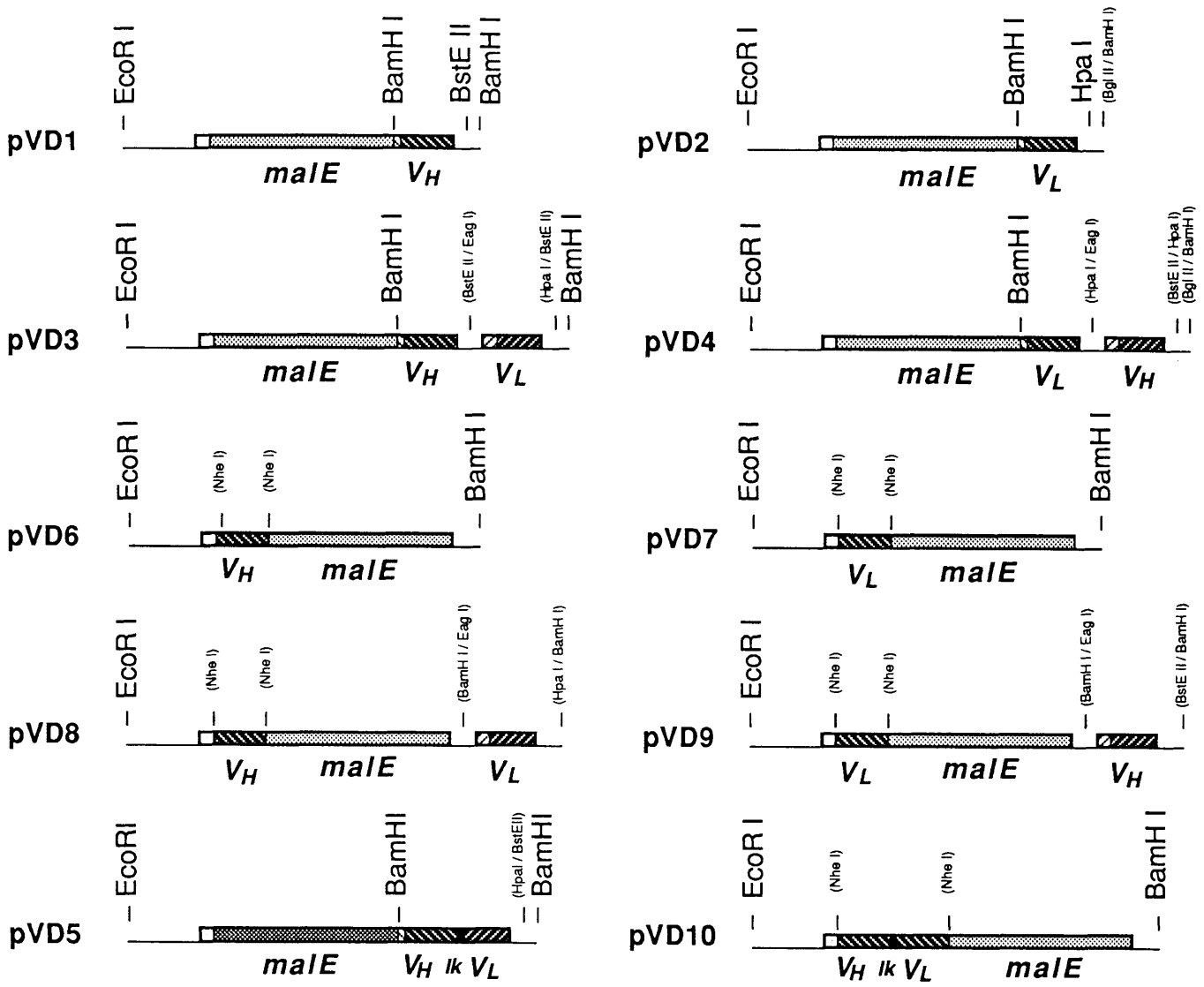

Fig. 1. Structures of the hybrid genes. The diagrams represent the engineered DNA fragments that were inserted between *Eco*RI and *Bam*HI sites of plasmid pPD140. Enzyme names in smaller characters and in brackets point to restriction sites that were altered by recombination or mutation. Boxes represent open reading frames. Signal sequences are indicated by clear fillings. V_H and V_L code for the V_H and V_L domains of antibody D1.3 and *lk* for a 15-residue peptide linker. The structures of pVD21, -31, -41, -81 and -91 were similar to those of pVD2, -3, -4, -8 and -9 respectively, except for the linker and intercistronic regions. The constructions are described in Materials and methods.

bacterial strains PD28(*recA*, Δ*malE444*, *malT*^{c}*1*) (Duplay *et al.*, 1987), HB2200 (*recA*, *malT*) (Bedouelle and Duplay, 1988) and HB2151*malT* (Martineau *et al.*, 1990), have all been described.

To minimize toxicity and instability problems, we propagated all the recombinant plasmids and phages in strains HB2200 or HB2151*malT*, in which the *malE* promoter is inactive (Bedouelle and Duplay, 1988).

Construction of chimeric genes

We fused individually the heavy chain and the light chain V-domains of antibody D1.3, V_H and V_L, to the C-terminus of protein MalE, in three steps at the genetic level. We first mutated the V_H and V_L genes by site-directed mutagenesis to create *Bam*HI restriction sites at the 3'-ends of their signal sequences. We then introduced stop codons immediately downstream from the last codons of the V_H and V_L genes. We finally inserted the *Bam*HI fragment that carried V_H or the *Bam*HI–*Bgl*II fragment that carried V_L, into the *Bam*HI site of plasmid pPD140. We thus obtained plasmids pVD1, coding for MalE–V_H and pVD2, coding for MalE–V_L (Figure 1). Both hybrid proteins were under the control of the signal peptide of MalE. As a result of these constructions, the tripeptide Ile–Leu–Ser was intercalated between MalE and V_H and the hexapeptide Ile–Pro–Gly–Ala–Arg–Cys between MalE and V_L. To test for a possible destabilization of the latter hybrid by a lone cysteine in the linker peptide, this residue was substituted for a serine by site-directed mutagenesis, resulting in plasmid pVD21.

We fused individually V_H and V_L with the N-terminus of MalE through the following steps. Since the cleavage of the signal peptide occurs between residues 26 and 27 of the MalE precursor, we created an *Nhe*I restriction site, overlapping codons 26 and 27 of the *malE* gene, by site-directed mutagenesis of phage M13mp18(*4am*)*malE* and obtained phage M13mp18(*4am*)*malE*(*NheI*). We then created *Nhe*I sites at the 5'- and 3'-ends of the V_H and V_L genes and inserted the resulting *Nhe*I fragments into M13mp18-*malE*(*NheI*). These insertions fused precisely the 3'-terminal codon of the MalE signal peptide to the 5'-terminal codons of the mature V-domains and added a few nucleotides between the 3'-terminal codons of the V-domains and the 5'-terminal codon of the mature MalE. As a result, MalE

was linked to V_H by the peptide Ala−Lys−Thr−Thr−Pro−Pro−Ala and to V_L by the peptide Ala−Asp−Ala−Ala−Ala. We then restored the residues of the V-domains and of MalE that had been altered when creating the *Nhe*I site and transferred the hybrid genes into plasmid pPD140 as *Eco*RI−*Bam*HI fragments. This gave plasmids pVD6 and pVD7 (Figure 1).

We created chimeric genes between the signal sequence of the *phoA* gene and either the V_H or V_L gene, as follows. We first introduced a *Bam*HI restriction site within the first codons of the mature protein PhoA, by site-directed mutagenesis. We then inserted the *Bam*HI fragment of the heavy chain cDNA that contained V_H or the *Bam*HI−*Bgl*II fragment of the light chain cDNA that contained V_L, into the *Bam*HI site created in *phoA*. The signal sequence of *phoA* was then fused precisely with the coding sequence of both V-domains by site-directed mutagenesis. The chimeric gene with V_H was carried by an *Eag*I−*Bst*EII fragment and the chimeric gene with V_L by an *Eag*I−*Hpa*I fragment, which both contained the ribosome-binding sequence of *phoA*, but not its transcriptional promoter.

We constructed dicistronic operons with one *V* gene fused to *malE* and the other one fused to the signal sequence of *phoA*, as follows. The *Eag*I−*Hpa*I and *Eag*I−*Bst*EII fragments, that coded for the V-domains fused with the PhoA signal peptide, were blunt-ended and inserted into the *Bst*EII site of pVD1, the *Hpa*I site of pVD2 and the *Bam*HI sites of pVD6 and pVD7. This gave plasmids pVD3, pVD4, pVD8 and pVD9 (Figure 1). In these constructs, the stop codon of the first cistron was distant from the initiator codon of the second cistron by 109 nucleotides (pVD4) or 127 nucleotides (others). Because these long stretches of nucleotides could impede the expression of the downstream cistrons, we substituted them by the sequence 5′-ATCGATGGA-GAAAATAAA-3′, using site-directed mutagenesis. This 18-base sequence is identical, except at its four 5′-positions, to the spacer between V_H and V_L in plasmid pASK30 (Skerra *et al.*, 1991), which allowed efficient expression of both V-domains. We established these constructs in derivatives of pTZ18R (Mead *et al.*, 1986) which we called pVD31, pVD41, pVD81 and pVD91.

We constructed hybrids MalE−scFv and scFv−MalE by joining the last codon of V_H with the first codon of V_L, through the sequence 5′-GGAGGCGGTGGCTCGGGCGGTGGTGGG-TCGGGTGGCGGCGGTTCT-3′, which codes for $(Gly_4-Ser)_3$ (Huston *et al.*, 1988). Thus, we obtained plasmid pVD5 by site-directed mutagenesis of pVD3 and plasmid pVD10 by site-directed mutagenesis of a recombinant between pVD6 and pVD7 (Figure 1).

Expression and purification of hybrid proteins

The media contained 100 μg/ml ampicillin. We grew all PD28 derivatives that contained pVD plasmids on plates of LB medium (10 g/l bactotryptone, 5 g/l yeast extract, 10 g/l NaCl) supplemented with 10 mg/ml glucose to repress the *malE* promoter. For the production of hybrid proteins, we first grew the PD28 derivatives overnight at 30°C. We collected the cells by centrifugation, then resuspended them in four times the initial volume of LB medium with 1 mg/ml glucose and incubated them for 7 h at 24°C.

We prepared periplasmic extracts as described (Bedouelle and Duplay, 1988), but for the following modifications. We performed the osmotic shock by resuspending the cells in 0.5 mM $ZnCl_2$, 0.5 mM phenylmethylsulphonyl fluoride (Clément and Popescu, 1991) and then adjusted the periplasmic fluid to 50 mM Tris−HCl, pH 7.5, 100 mM NaCl. The shocked cells were frozen, thawed and resuspended either in cracking buffer (Laemmli, 1970) for direct electrophoresis or in membrane buffer (10 mM Tris−HCl, pH 8.5, 5 mM EDTA, 200 mM KCl, 5 mM β-mercaptoethanol) for sonication. Sonicated extracts were centrifuged for 1 h at 100 000 *g* and 4°C (Silhavy *et al.*, 1976). The insoluble and membrane-associated materials were resuspended in membrane buffer. All the extracts were adjusted to 1/10 of the volume of the original bacterial culture.

The hybrids were purified from periplasmic extracts by affinity chromatography on a column of cross-linked amylose, as described (Blondel and Bedouelle, 1990). Full-length hybrids were separated from incomplete products by ion-exchange chromatography, using a Pharmacia FPLC system and a Mono-Q HR 5/5 column (Pharmacia). The samples were applied to the column in 30 mM Tris−HCl, pH 7.6, 20 mM NaCl. Then, a 20−220 mM gradient of NaCl was run over 15 column volumes. In these conditions, single-chain hybrids and heterodimers were eluted at 75 mM NaCl and the main peak of incomplete products at 100 mM NaCl.

Electrophoresis and Western blotting of proteins

Polyacrylamide gel electrophoresis (SDS−PAGE) and electrotransfer to Hybond C nitrocellulose sheets (Amersham) were performed as described (Bedouelle and Duplay, 1988; Sambrook *et al.*, 1989). The immobilized hybrid proteins were revealed with a rabbit antiserum directed against MalE, as a first reagent and with a goat monoclonal antibody, directed against the Fc fragment of rabbit immunoglobulin and conjugated to alkaline phosphatase, as a second reagent.

ELISA

We performed the ELISA tests as described (Ward *et al.*, 1989a). The wells of the microtitration plates were coated with 100 μl of a solution of antigen at 2−4 μg/ml. Hybrids were monitored using the same immunological reagents as for Western blots. Native D1.3 was monitored with a goat antibody conjugate, specific for mouse immunoglobulin.

Equilibrium dissociation constants

The equilibrium dissociation constants (K_D) between the hybrids and HEL were determined by competition ELISA as described (Friguet *et al.*, 1989). The hybrid proteins were incubated in solution with various concentrations of HEL, overnight at 4°C. Then the remaining, free binding sites were titrated by ELISA at 4°C. The values of K_D were determined graphically (Easson and Stedman, 1936; Friguet *et al.*, 1989). For divalent molecules, the calculations were adapted according to Stevens (1987).

Results

Design of hybrid proteins

We fused the variable domains, V_H and V_L, of antibody D1.3 with protein MalE by site-directed mutagenesis and genetic engineering. V_H and V_L were appended to the N-terminus and to the C-terminus of MalE and the hybrid proteins were set under the control of the signal peptide of MalE. In parallel, we fused V_H and V_L with the signal peptide of the alkaline phosphatase (PhoA) of *E.coli*. The resulting genes were assembled in transcriptional units under the control of the *malE* promoter on plasmids derived from pBR322, so that a 5′-cistron coded for a hybrid between MalE and a V-domain and a 3′-cistron coded for the other V-domain under the control of the PhoA signal peptide. Finally, we fused the C-terminus of V_H with the N-terminus of V_L through a 15-residue peptide linker to obtain single-chain variable fragments (scFv). The details of these constructions are given in Materials and methods, the structures

of the recombinant genes in Figure 1, and the nomenclature of the hybrids in Table I.

Expression and toxicity of the hybrids

To produce the hybrid proteins, we transferred the recombinant plasmids into PD28, a strain which constitutively expresses the maltose regulon but cannot ferment maltose because it harbours deletion Δ*malE*444 (Duplay *et al.*, 1984). In order to test the expression of the hybrids, their ability to transport maltose and their toxicity to the host cell, we streaked the PD28 derivatives at 25, 30 and 37°C on McConkey indicator plates supplemented with 1% maltose. The results are given in Table I. They showed that several hybrid proteins were produced and that at least their MalE portion was exported to the periplasm and was active in maltose transport. Plasmid pVD10, that coded for scFv−MalE, was toxic to PD28 at all temperatures. The other plasmids were not toxic at 25°C, although some of them were so at 30 or 37°C.

Table I. Phenotypes of the PD28 derivatives at various temperatures[a]

Plasmids	Hybrids[b]	37°C[c]	30°C[c]	25°C[c]
pBR322	none	−	−	−
pPD1	wild type MalE	+	+	+
pVD1	MalE−V_H	−	−	−
pVD2	MalE−V_L	−	+/−	+/−
pVD3	MalE−V_H::V_L[d]	−,*t*	+	+
pVD4	MalE−V_L::V_H[d]	+	+	+
pVD5	MalE−scFv	−,*t*	+	+
pVD6	V_H−MalE	−	−	−
pVD7	V_L−MalE	−	−	−
pVD8	V_L::V_H−MalE[d]	−,*t*	−,*t*	+
pVD9	V_H::V_L−MalE[d]	−,*t*	+	+
pVD10	scFv−MalE	−,*t*	−,*t*	+/−,*t*

[a]Colonies were streaked at three different temperatures onto pre-warmed McConkey indicator plates supplemented with 1% maltose.
[b]MalE−*x* and *x*−MalE represent *x* fused to the C-terminus and to the N-terminus of MalE respectively.
[c]'+', red colonies, indicating the ability of the plasmid to complement deletion Δ*malE444* of PD28 with maltose fermentation; '−', white colonies, indicating the inability to ferment maltose; '+/−', pink colonies; '*t*', very small colonies, indicating a toxicity of the plasmid.
[d]We have no evidence that free V_H and V_L domains were expressed from the dicistronic operons carried by plasmids pVD3, -4, -8 and -9, as they were from plasmids pVD31, -41, -81 and -91.

Identification and cellular location of the hybrids

To visualize the hybrids and determine their cellular location, we submitted the producing bacteria to a cold osmotic shock (Neu and Heppel, 1965) and analysed the periplasmic fluids and shocked cells by gel electrophoresis and Western blots. The results are shown in Figure 2. Most PD28 derivatives produced proteins that reacted with an anti-MalE serum and had the apparent mol. wts expected for the hybrids. The full-length hybrids were contaminated with degraded or abortive products, most of them heavier than MalE, as already described for other hybrids (Blondel and Bedouelle, 1990; Clément and Popescu, 1991). The proportion of these incomplete molecules varied with the hybrid and was reduced when the D1.3 domain was fused with the N-terminal end of MalE. All the hybrids that we found in the periplasmic fraction were also present in the shocked cells, in ~5-fold higher amounts and less contaminated with incomplete molecules. Polypeptides that were slightly larger than the full-length hybrids were found in the fraction of the shocked cells and could be precursors with uncleaved signal peptides (Figure 2b). After sonication and centrifugation of the shocked cells, most of the full-length hybrids sedimented with the insoluble materials (Figure 2c). Staining with Coomassie blue revealed that few other proteins were present in this fraction (not shown). The production of free V-domains from the dicistronic operons was not investigated in this experiment.

Production of antigen-binding activity and growth conditions

We detected the presence of HEL-binding activity in the periplasm of the PD28 derivatives and in the culture media, by ELISA tests as described in Materials and methods. We monitored the production of antigen-binding activity by PD28(pVD5) and PD28(pVD10), used as pilot strains, in experiments where we varied the growth conditions to improve the yields. First, the cells produced more activity when grown at 24°C rather than at 30°C or above. A similar effect of temperature has been described for the production of free Fv and Fab fragments (Skerra and Plückthun, 1991). Second, the activity increased with cell density up to the stationary phase and harvesting the cells at early rather than late stationary phase minimized the accumulation of incomplete products (not shown). Third, the yields of HEL-binding activity depended on the sugar supplies as follows: (i) the addition of glucose at 10 g/l repressed the production of antigen-binding activity; (ii) glucose at 1 g/l gave no repression, but strongly

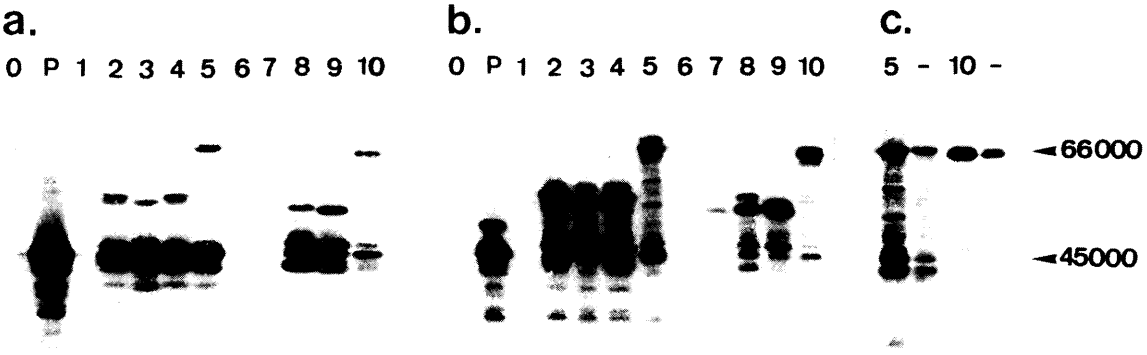

Fig. 2. Cellular locations of the hybrids. The periplasmic extracts, shocked cells and insoluble cellular materials were prepared from PD28 derivatives harbouring the coding plasmids. All extracts were adjusted to 1/10 of the volume of the original bacterial culture. They were analysed by Western blotting using an anti-MalE serum. The samples were loaded as follows: (**a**) 5 μl of periplasmic extracts, (**b**) 1.5 μl of whole shocked cells, (**c**) 5 μl and 1.5 μl of resuspended 10 000 × *g* pellets. The numbers indicate the rank of the tested plasmids in the pVD series: 0, no plasmid; P, parental plasmid pPD140 (with no insert). The mol. wt markers were not revealed by the anti-MalE antibodies; their migrations are labelled in daltons.

reduced the release of activity into the culture medium, thereby improving its recovery from the periplasm; (iii) by contrast, maltose at 10 g/l favoured the release of activity into the culture medium and reduced the toxic effect of some hybrids at 30°C; (iv) combining glucose at 1 g/l and maltose at 1 g/l gave the best yields of activity in the periplasmic extracts, yet these conditions prevented the affinity purification of the hybrids on cross-linked amylose, probably because they remained bound to maltose molecules picked from the medium.

Purification of the hybrids

We purified the hybrids from periplasmic extracts of the producing strains by affinity chromatography on cross-linked amylose as described (Bedouelle and Duplay, 1988). MalE−V_L, V_H−MalE and V_L−MalE were expressed from pVD2, -5 and -6 and were eluted from the amylose column together with major amounts of incomplete molecules (not shown). These eluted fractions were used for the binding assays reported in the following sections. Hybrid MalE−V_H was produced in very small amounts from pVD1 and was not purified.

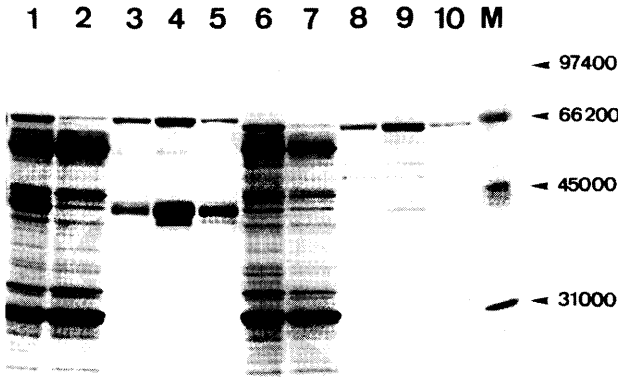

Fig. 3. Affinity chromatography. Periplasmic extracts, prepared from strains PD28 (pVD5) (lanes 1−5) and PD28 (pVD10) (lanes 6−10) were subjected to one run of chromatography on a column of cross-linked amylose. The purified fractions were analysed by SDS−PAGE and the gels stained with Coomassie blue. Lanes 1 and 6, crude periplasmic extracts; lanes 2 and 7, flow-through fractions; lanes 3−5 and 8−10, successive eluted fractions; M, mol. wt markers, labelled in daltons.

Figure 3 illustrates the purification of single-chain hybrids. MalE−scFv co-purified with at least an equivalent amount of incomplete products (lanes 3−5), while these were much less abundant with scFv−MalE (lanes 8−10). The components of the purified fractions were present in the crude periplasmic fluid (lanes 1 and 6), but not in the flow-through of the affinity column (lanes 2 and 7), which indicated that most hybrid molecules bound amylose. We obtained ~1 mg of full-length hybrid from 1 l of culture at A_{600nm} = 2.5 cm^{-1}. This corresponds to the purification of 5000 full-length molecules from the periplasm of each producing cell.

When we tried to purify heterodimers from PD28 derivatives harbouring plasmids pVD3, -4, -8 and -9, we did not detect the free V-domains in the purified fractions by SDS−PAGE and Coomassie blue staining. We therefore constructed plasmids pVD31, -41, -81 and -91, identical to the previous ones but with a shorter intercisteronic region (Materials and methods). We were able to produce and to purify heterodimers from strains harbouring these plasmids (Figure 4a), which showed that pVD3, -4, -8 and -9 did not express the free V-domains because of inadequate intercistronic regions.

We purified the heterodimers and obtained similar yields as for MalE−scFv and scFv−MalE. Figure 4a shows such affinity-purified fractions. The purified heterodimers, like the single-chain hybrids, were contaminated with various amounts of incomplete molecules. Again, this phenomenon was limited when the D1.3 domain was fused with the N-terminal end of MalE rather than with its C-terminal end. In the particular case of pVD91, which codes for the heterodimer V_H::V_L−MalE, we obtained pure heterodimer directly from the amylose column.

When necessary, intact hybrids were separated from the contaminants by ion-exchange chromatography (Materials and methods). Figure 4b and c illustrates the purification to homogeneity of MalE−scFv and MalE−V_H::V_L, starting from fractions where the relative amounts of these hybrids were <5%. Two pieces of evidence showed that most MalE−V_H and V_L were associated in heterodimers: (i) the two polypeptides were co-eluted in a sharp peak from the ion-exchange column although their theoretical isoelectric points are quite different (5.36 and 9.23 respectively); (ii) when this pure fraction was analysed by

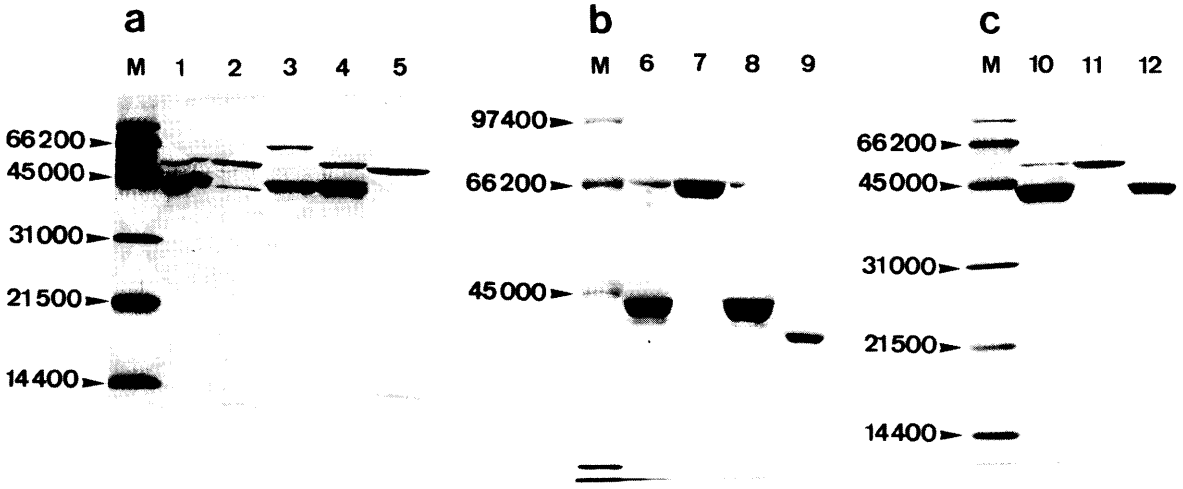

Fig. 4. Purification of hybrids to homogeneity. Samples of hybrids were subjected to SDS−PAGE after different steps of purification. The gels contained (**a**) 17%, (**b**) 10% or (**c**) 15% of acrylamide and were stained with Coomassie blue. (**a**) Fractions purified by one step of chromatography on cross-linked amylose, containing the following hybrids: 1, MalE−V_H::V_L; 2, V_L::V_H−MalE; 3, MalE−scFv; 4, MalE−V_L::V_H; 5, V_H::V_L−MalE. (**b**) and (**c**) Fractions obtained after an additional step of chromatography on a Mono-Q column. (**b**) Purification of MalE−scFv from the sample analysed in lane 3. (**c**) Purification of MalE−V_H::V_L from the sample of lane 1. Lanes 6 and 10, samples before purification; lanes 7 and 11, purified hybrids; lanes 8 and 12, main species of incomplete products; lane 9, pure, wild type MalE; M, mol. wt markers, labelled in daltons.

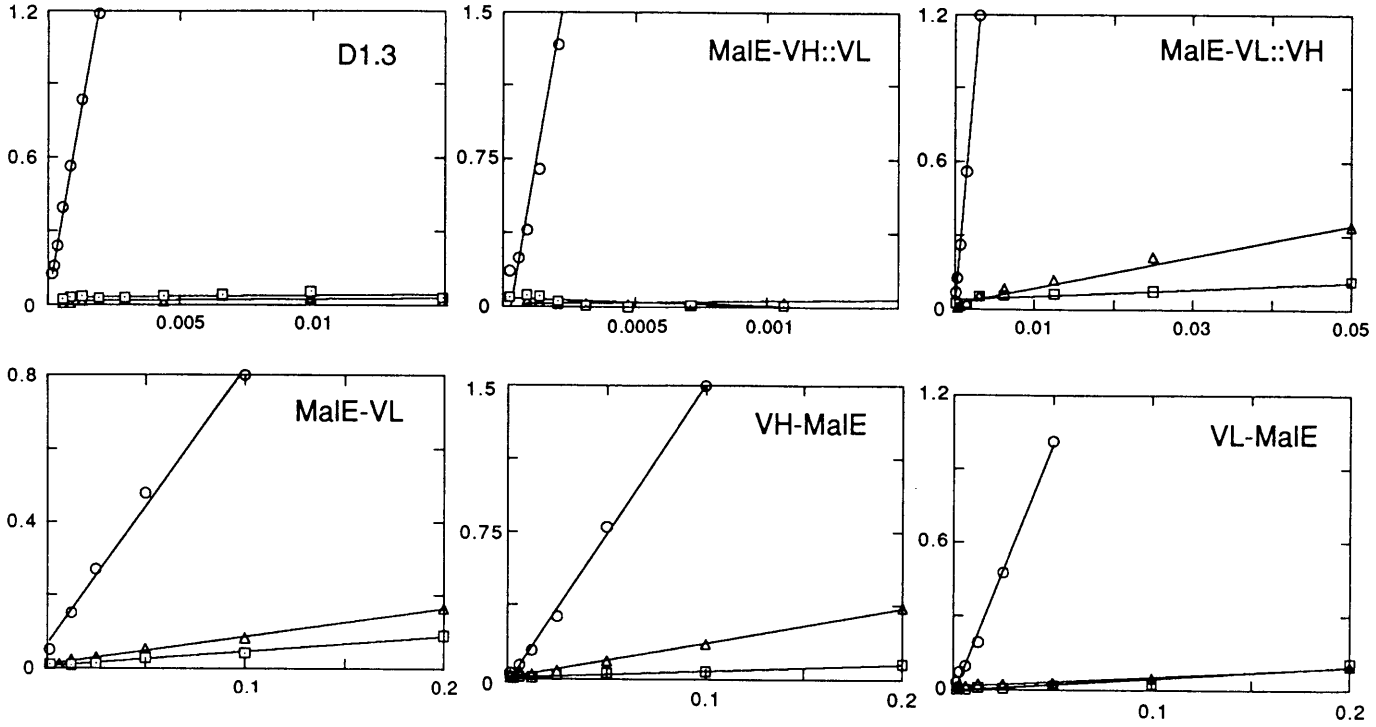

Fig. 5. Specificity of binding. Purified hybrids and a crude ascite fluid containing antibody D1.3, were assayed in ELISA tests. Binding was measured as a colorimetric signal at 420 nm and was plotted versus the dilution of the fractions under test. The undiluted samples contained different concentrations of hybrids. V_H-MalE, V_L-MalE and $MalE-V_L$ were obtained from plasmids pVD2, -6 and -7 respectively. $MalE-V_H$ was not tested because it was poorly produced by plasmid pVD1. Codes for the coating antigens: ○, HEL; △, TEL; □, BSA. The straight lines were fitted to the experimental data with the program Kaleidagraph™.

SDS−PAGE, it gave two bands with relative intensities that were consistent with the stoichiometric ratio.

Specificity of binding

We compared the binding of purified hybrids to HEL, turkey egg white lysozyme (TEL) and bovine serum albumin (BSA) in direct ELISA (Figure 5). TEL was a pertinent specificity control because its amino acid sequence differs from that of HEL by only seven amino acids and yet it cross-reacts only weakly with antibody D1.3 (Harper *et al.*, 1987). Gln121, which belongs to the epitope of D1.3 (Bhat *et al.*, 1990), is replaced by His in TEL. BSA was chosen as an aspecific control. The ratios of the slopes corresponding to HEL binding versus TEL or BSA binding were taken as specificity coefficients for each hybrid (Table II). The pattern of these specificity coefficients was reproducible although their individual values varied slightly in independent experiments. They can therefore be considered as semi-quantitative indicators.

The results of Table II show that the single-chain hybrids and the heterodimers were highly specific for HEL, except $MalE-V_L::V_H$. This heterodimer bound the HEL-coated wells relatively poorly (e.g. 40-fold less than $V_H::V_L-MalE$ at the same concentration), which suggested that parts of its molecules were inactive. The hybrids between MalE and single V-domains retained a significant specificity for HEL. This specificity was higher for V_L than for V_H when the two domains fused at the N-terminus of MalE.

Affinity for the antigen

We determined the equilibrium dissociation constants (K_Ds) between the purified hybrids and HEL by competitive ELISA (Friguet *et al.*, 1989). Various concentrations of HEL were equilibrated overnight with the hybrids and the free antigen-binding sites were then titrated by ELISA. The K_Ds were determined in parallel by Klotz plots as described (Friguet *et al.*, 1989) and by Easson plots (Easson and Stedman, 1936). The results are shown in Figure 6 and in Table III. The heterodimers had K_Ds 3.5- and 10-fold lower than native D1.3, except $MalE-V_L::V_H$ which had a higher K_D. The hybrids MalE−scFv and scFv−MalE had K_Ds of the same order as native D1.3.

We could not determine K_Ds for V_L-MalE or $MalE-V_L$, because these hybrids bound HEL erratically after overnight equilibration with competitor HEL. They also bound TEL and BSA. This behaviour could be due to a low stability and a partial denaturation of the single V_L domain. V_H-MalE remained stable and specific in the same experimental conditions. Its K_D was only 2-fold larger than that of native D1.3, and 20-fold larger than that of the corresponding heterodimer $V_L::V_H-MalE$. These differences were surprisingly small since this hybrid contained only one half of the D1.3 binding site.

Table II. Specificity coefficients[a]

Hybrid	HEL/TEL	HEL/BSA
Native D1.3	230	>500
$MalE-V_H::V_L$[b]	>500	>500
$MalE-V_L::V_H$[b]	60	270
$V_L::V_H-MalE$[b]	>500	>500
$V_H::V_L-MalE$[b]	>500	>500
MalE−scFv	370	>500
scFv−MalE	>500	>500
$MalE-V_L$	6	14
V_H-MalE	7	28
V_L-MalE	38	37

[a]The specificity coefficients were defined as the ratios of the slopes corresponding to HEL binding versus TEL or BSA binding, in Figure 5 and in similar graphs.
[b]The heterodimers were expressed from plasmids pVD31, -41, -81 and -91.

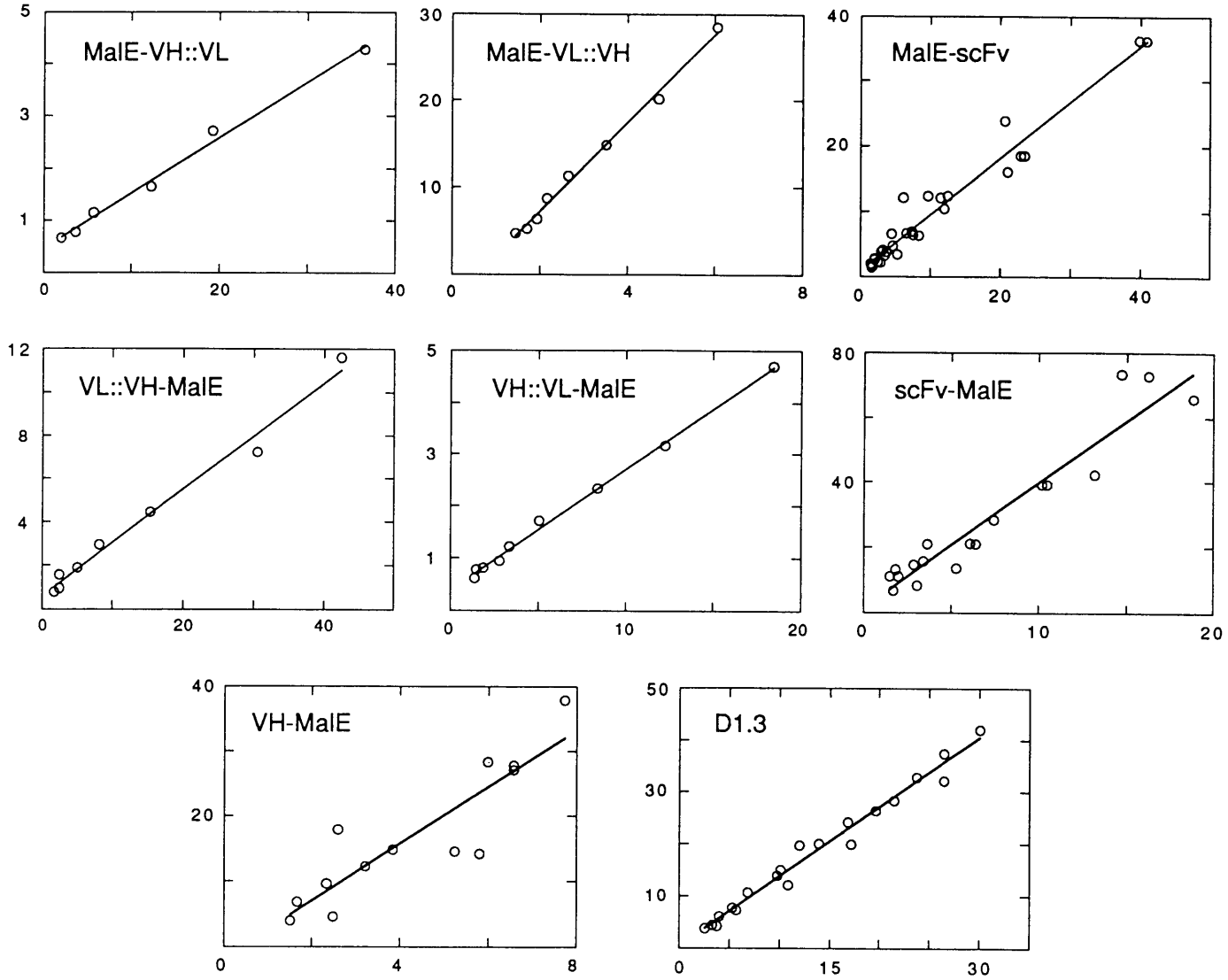

Fig. 6. Determination of equilibrium dissociation constants. Competition ELISAs were performed with affinity-purified hybrids or a crude ascite fluid containing antibody D1.3. The figure represents Easson plots of the experimental data (Easson and Stedman, 1936). If c is the concentration of antigen, a the amount of bound hybrid or antibody for a given value of c and a_0 the value of a when $c = 0$, the proportion of free binding sites is expressed by $i = (a_0 - a)/a_0$. The Easson plots represent c/i (nM) as a function of $1/(1 - i)$. The straight lines were fitted to the data with the program Kaleidagraph™ and the K_Ds are given by their slopes. Hybrid V_H–MalE was expressed from plasmid pVD6.

Discussion

MalE as a vector for the production and purification of active antibody fragments

In this work, we linked the V-domains of antibody D1.3 to the N-terminus and to the C-terminus of MalE and produced hybrid proteins between MalE and either a single-chain Fv or a heterodimeric Fv or single V-domains. These hybrids were purified on cross-linked amylose and obtained free of degraded or abortive polypeptides, directly or after an additional step of chromatography. They bound the antigen specifically, which showed that their binding sites were accessible to HEL, a protein of 14 400 Da. These results extend the previous finding that hybrids between MalE and foreign proteins generally keep the activities of both partners (Bedouelle and Duplay, 1988; Maina *et al.*, 1988; Blondel and Bedouelle, 1990; Szmelcmann *et al.*, 1990) and qualify MalE as a vector to produce, purify and assay antigen-binding fragments.

Purification of hybrids with MalE depends neither on the properties of the antibody fragment, nor on the availability of the anti-idiotype antibody, so that it can apply to mutants with altered binding properties. In principle, fusions with an epitope ('peptide tag'), a streptavidin-binding peptide ('*Strep*-tag') or an

Table III. Equilibrium dissociation constants (nM)[a]

Hybrid	Easson plots	Klotz plots
Native D1.3	1.0	1.3
MalE–V_H::V_L[b]	0.10	0.14
MalE–V_L::V_H[b]	4.7	3.0
V_L::V_H–MalE[b]	0.25	0.40
V_H::V_L–MalE[b]	0.22	0.34
MalE–scFv	0.70	1.0
scFv–MalE	3.0	2.0
V_H–MalE	4.5	2.0

[a]The K_Ds corresponding to HEL binding by the D1.3 antibody and by the hybrids listed in column 1, were determined in parallel by Easson and Klotz plots as described in Materials and methods. The incertitude was estimated to be within a factor of 2; it was slightly higher for V_H–MalE because this hybrid reproducibly gave more dispersed values.
[b]The heterodimers were expressed from plasmids pVD31, -41, -81 and -91.

oligoaminoacid (such as oligo-His) should offer similar advantages (Hochuli *et al.*, 1988; Hopp *et al.*, 1988; Martin *et al.*, 1990; Skinner *et al.*, 1991; Schmidt and Skerra, 1993). In practice, however, proteins fused with polyaminoacids are retained on adsorbent columns with relatively poor selectivity (Uhlèn *et al.*, 1992). Proteins fused with peptide- or *Strep*-tags are purified on columns of immobilized antibody or streptavidin, which are expensive, can be regenerated only a limited number of times and get easily degraded. Proteins fused with polyaminoacids or peptide tags are eluted from the affinity columns in rather harsh, denaturing conditions (discussed in Schmidt and Skerra, 1993).

In this context, MalE offers a number of advantages when used as an affinity handle.

(i) The cross-linked amylose column is stable and can be reused indefinitely (for more than 7 years in our laboratory).
(ii) The conditions of elution are mild and non-denaturing.
(iii) MalE is highly antigenic and facilitates the detection of the linked protein or antibody fragment: the HEL-binding activities of the hybrids between MalE and Fv or scFv, were detected by ELISA in crude periplasmic extracts or in culture supernatants, after a 100-fold dilution or more (not shown).
(iv) MalE may contribute to the stability or activity of the linked V-domains, as suggested by the fact that we were able to purify hybrids between MalE and V_L that bound HEL, whereas the free V_L domain of D1.3 cannot be purified by immunoadsorption on immobilized HEL (Ward *et al.*, 1989b).

Production and purification of hybrids with Fv and scFv

The hybrids with single-chain and heterodimeric Fvs were expressed at similar yields, provided that an appropriate intergenic sequence allowed the second cistron of the dicistronic operons to be transcribed. The heterodimers remained stable during purification by either affinity chromatography on cross-linked amylose or ion-exchange chromatography, as shown by the presence of the hybrids and the free V-domains in approximately stoichiometric amounts in the purified fractions.

The hybrids were contaminated with various amounts of incomplete products, depending on their fusion patterns with MalE (see Figures 2–4). We did not attempt to use the protease-deficient strains *ompT*, *lon* or *degP*, because in the case of hybrids with MalE, these mutants do not significantly improve the recovery of full-length molecules (Clément *et al.*, 1991). When the V-domains were placed at the C-terminal end of MalE, the incomplete products represented 60–95% of the affinity-purified materials. When the same domains were positioned at the N-terminal end, however, the proportion of these contaminants was limited to <25% or eliminated, depending on the hybrid configuration. These results suggest different possibilities: (i) fusion with MalE may disturb the native conformation of the V-domains and make them sensitive to proteolytic degradation in some constructions, (ii) some of the oligopeptide linkers that we used for the fusions may induce proteolysis, (iii) production of incomplete polypeptides may be due to abortive translation, rather than to degradation: then, truncated hybrids would be made that lack their C-terminal end.

The optimal conditions were met with the heterodimer V_H::V_L–MalE, where V_L was fused to the N-terminus of MalE through the pentapeptide Ala–Asp–Ala–Ala–Ala. This molecule was recovered free of incomplete products from the amylose column, at a yield of 1.2 mg/l culture medium.

Binding properties of fused Fv and scFv

The K_Ds that corresponded to HEL binding of the heterodimeric hybrids, except MalE–V_L::V_H, were lower than that of native D1.3 (Table III). The K_D ratios between the heterodimers and D1.3 were 0.1 for MalE–V_H::V_L and ~0.4 for both V_H::V_L–MalE and V_L::V_H–MalE, corresponding to free energies of −1.3 and −0.76 kcal/mol respectively. Since such an enhancement of affinity does not exist for the free Fv (Ward *et al.*, 1989a), these results show that MalE stabilized the interaction between the V-domains of D1.3 and HEL.

MalE–V_L::V_H had lower affinity and specificity for HEL than the other heterodimeric hybrids and than native D1.3 (Tables II and III). This indicated the existence of unfavourable interactions between MalE and V_L when the latter was fused at the C-terminus of MalE. This was further suggested by the fact that hybrid MalE–V_L had a lower specificity than V_L–MalE. We checked that this difference was not due to the presence of a lone cysteine residue in the linker peptide between MalE and V_L: changing this cysteine to a serine enhanced the production of MalE–V_L by 2-fold, but left its binding properties unchanged (not shown).

MalE–scFv and scFv–MalE had K_Ds in the same range as native D1.3 (Table III). This shows that the N-terminal ends of V_H and V_L can be blocked simultaneously, as they were in MalE–scFv, without hindering the recognition of a large antigen like lysozyme. The affinities of the two hybrids were reduced 7- to 9-fold when compared to the corresponding heterodimers, which suggests that the peptide linker between V_H and V_L either slightly disturbed HEL binding, thus counterbalancing the contribution of MalE, or prevented the action of MalE.

The K_Ds of MalE–scFv and MalE–V_H::V_L were ~3-fold lower than those of scFv–MalE and V_L::V_H–MalE, showing that the contribution of MalE to HEL binding energy was larger when it was connected to the N-terminal end of V_H, closer to the antigen-binding site.

Binding properties of fused single V-domains

The hybrids between MalE and single V-domains specifically bound HEL, compared to TEL and BSA, although with a lower specificity than native D1.3 (Table II). This showed that each V-domain, when fused with MalE, recognized the antigen and discriminated by itself between HEL and TEL. So far, only V_H domains have been reported to do this (Ward *et al.*, 1989a). V_L–MalE achieved this discrimination better than V_H–MalE, consistently with the crystal structure of the complex between D1.3 Fv and HEL (Bhat *et al.*, 1990). Indeed, residue Glu121 of HEL, which is replaced by His in TEL, interacts with four residues of V_L (Tyr32, Phe91, Trp92 and Ser93) and with only one residue of V_H (Tyr101). This consistency with structural data suggests that, in our experiments, HEL formed interactions with unpaired V_H and V_L that exist in its complex with native D1.3. Thus, the observation that single light-chain V-domains do not bind the antigen (Field *et al.*, 1989) should not be considered as general. Kabat and Wu (1991) have proposed that the antibody-forming process relies more strongly on V_H than on V_L and Collet *et al.* (1992) have shown that the specificities of a series of antibodies are determined by the heavy chain and not by the light chain. In contrast, our results show that the unpaired V_L domain of D1.3 can contribute to the specificity of the native antibody.

We determined the K_D of V_H–MalE by two different graphical methods and found values of 4.5 nM and 2.0 nM (Table III). The K_D of the isolated V_H is 19 nM (Ward *et al.*,

1989a). Thus, as in the case of the hybrids with Fv (see previous paragraph), we observed a K_D decrease between the free V_H and V_H−MalE, which was probably due to a contribution of MalE to HEL binding. The K_D ratio was 0.15, corresponding to a free energy of −1.0 kcal/mol. Hence, the contribution of MalE to binding was comparable in V_H−MalE and in the heterodimers.

The K_D ratio between the single V_H domain and the V_L::V_H heterodimer is ~10-fold, whether comparing V_H−MalE and V_L::V_H−MalE (Table III) or comparing the isolated V_H and the native D1.3 (Ward *et al.*, 1989a). This factor is unexpectedly small, since half of the antigen-binding site is missing in the single V_H. Ward *et al.* (1989a) have proposed that in the absence of V_L, the surface of interaction between V_H and HEL reorientates slightly and creates a new set of contacts. This hypothesis may well be correct and the contribution of V_H to the binding energy would then be different when it binds alone and when it does so as a part of the V_L::V_H heterodimer. The contribution of V_L to HEL binding to native D1.3 should therefore not be evaluated from the difference between the affinities of the single V_H and of the heterodimer for HEL. In point of fact, our results on HEL binding by MalE−V_L and V_L−MalE strongly suggest that the contribution of V_L to the binding energy of D1.3 is significant.

Two recent publications on the V_H domain of D1.3 have brought interesting data for this discussion. Berry and Davies (1992) have shown that HEL can be retained on a column of immobilized V_H and then be released during a one-step elution at 4 M $MgCl_2$ (pH 7). Because TEL is retained and released in the same conditions, they claim that isolated V_H does not discriminate between HEL and TEL and, hence, is poorly specific. However, they did not determine whether HEL and TEL are eluted by the same or by different concentrations of $MgCl_2$, which makes their conclusion questionable. Boorebaeck *et al.* (1992) have reported that the affinity of V_H for HEL is much lower than those of Fv and Fab when the measures are made in solid phase with a Bioacore biosensor (Pharmacia). These experimental conditions, however, do not reflect the equilibrium association in aqueous phase, as shown by the important differences in affinity they find between Fv, Fab and the native antibody. Furthermore, in both published studies, the V_H domains were prepared by affinity chromatography on a HEL-Sepharose column, a method that requires semi-denaturing conditions for elution. The proportion of native V_H molecules in the purified samples could therefore be lower than expected and the affinity and specificity assays be affected. In our work, the affinities were determined in aqueous phase by a method that does not depend on the concentration of binding molecules. This can account for discrepancies between our results and those discussed.

Contribution of MalE to HEL binding: qualitative aspects

We purified hybrids between MalE and V_L that bound HEL, whereas the free V_L domain of D1.3 cannot be purified by immunoadsorption on immobilized HEL (Ward *et al.*, 1989b). This suggests that MalE stabilized V_L in an active form, perhaps by preventing its spontaneous dimerization as described for other light chain V-domains (Plückthun, 1991).

More generally, the biochemistry of V-domains is difficult because they are relatively unstable (Huston *et al.*, 1988; Ward *et al.*, 1989a; Plückthun, 1992). They have hydrophobic surfaces exposed to the solvent, tend to adhere to hydrophobic ligands and are poorly soluble as monomers. The surface of MalE carries hydrophobic residues in the vicinity of the maltose- and maltodextrin-binding site, as well as areas of interactions with several proteins involved in the transport of these sugars and in chemotaxis towards them (Spurlino *et al.*, 1991). Hydrophobic surfaces of MalE and of the V-domains might interact within the hybrids and stabilize the structures of unpaired V-domains, thus restricting their 'hydrophobic stickiness' and making their use easier in biochemical studies.

The enhancement of hybrid affinity for HEL is unlikely to be related to V-domain stabilization, because it was observed in most heterodimeric hybrids, where the two V-domains are mutually stabilized already. We propose therefore that MalE can make direct, unspecific contacts with bound HEL and, thus, contribute to the energy of binding without spoiling the overall specificity of interaction. The potential binding of MalE to HEL depended on the presence of the V-domain, since MalE alone did not bind HEL in direct ELISA (not shown).

Concluding remarks

Fusion with MalE appears a valuable tool to investigate the mechanisms by which antibody fragments bind their antigens, in particular in the case of D1.3 and HEL. It remains to be shown whether antibody fragments of diverse origins and specificities keep their activity when fused with MalE. If so, advantage could be taken of the stabilization of single V-domains by MalE to screen libraries of such hybrids for antigen-binding. Finally, hybrids between MalE and Fvs could be used for immunodetection or to immobilize antigen-binding or catalytic antibody fragments on surfaces covered with amylodextrins.

Acknowledgements

We thank Dr Greg Winter for the gift of recombinant phages carrying DNA copies coding for the V_H and V_x domains of antibody D1.3, Dr Guy Bordenave for helping us to prepare a rabbit antiserum directed against MalE, Dr Roberto Poljak for the gift of D1.3 ascite supernatant, Nicole Expert-Besançon for advice and help in FPLC chromatography and an anonymous referee for pertinent suggestions. This work was supported in part by the Ligue Nationale Française contre le Cancer and by the Comité Consultatif des Applications de la Recherche de l'Institut Pasteur. The first characterization of MalE−scFv hybrids has been published in a preliminary report (Brégégère and Bedouelle, 1992).

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Received February 4, 1993; revised September 2, 1993; accepted September 24, 1993