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Bacterial Expression and Secretion of Various Single-chain Fv Genes Encoding Proteins Specific for a *Salmonella* Serotype B O-Antigen*

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Active single-chain Fv molecules encoded by synthetic genes have been expressed and secreted to the periplasm of *Escherichia coli* using the *ompA* secretory signal. Four different constructs were developed to investigate the effects of peptide linker design and V_L-V_H orientation on expression, secretion, and binding to a *Salmonella O*-polysaccharide antigen. Peptide linker sequences derived from the elbow regions of the Fab molecule were used alone or in combination with the flexible $(GGGGS)_2$ sequence. V_L and V_H domain order in the single chain molecules had a profound effect on the level of secretion but hardly influenced total expression levels, which were ~50 mg/liter, chiefly in the form of inclusion bodies. With V_L in the NH_2-terminal position, the amount of secreted product obtained was 2.4 mg/liter, but when V_H occupied this position the yield was less than 5% of this value. Enzyme immunoassays of the four products showed domain order and linker sequence affected antigen binding by less than an order of magnitude. Attempts to express active Fv from dicistronic DNA were unsuccessful, but active Fv was obtained from single-chain Fv by enzymic cleavage at a site in the elbow linker peptide. The thermodynamic binding parameters of intact and cleaved single-chain Fvs determined by titration microcalorimetry were similar to those of bacterially produced Fab and mouse IgG.

The antigen-binding site of an antibody is formed by the non-covalent association of the variable domains (V_L[1] and V_H) at the amino termini of the heavy and light chains. For *in vivo* diagnostic and immunotherepeutic applications, small antibody fragments are desirable because of reduced immunogenicity and shorter tissue clearance times (31). Fv fragments consisting of the V_L and V_H domains alone are such molecules, but attempts to isolate Fv fragments by proteolytic digestion of the intact antibody are rarely successful.

Recently, the production of antigen-binding proteins by recombinant DNA technology has opened up new avenues to build novel antibody-based molecules useful for the treatment of human diseases (1). The most interesting of such molecules produced thus far are single-chain Fvs (sFvs) in which the COOH terminus of one variable domain is joined to the NH_2 terminus of the second variable domain by a linker of appropriate length and flexibility. Linking the domains in this way results in a stable, active molecule and overcomes possible problems with V_H-V_L dissociation at low concentration (2). Following the first reports of the production of these molecules with either V_L or V_H in the amino-terminal position (3, 4), sFvs blocking rhinovirus infection (5) and sFvs linked to a toxin to form immunotoxins directed against ovarian cancer cells (6) have been described. Single-chain Fvs have also been useful in studying structure-function relationships in the V_L and V_H domains (2, 7) and in expressing antibody variable domains as fusion proteins on bacteriophage surfaces for the purpose of screening for antigen binding (8).

Typically, linkers designed from simple amino acid sequences such as $(GGGGS)_3$ have been used, but in one report (5) linkers incorporating an interdomain sequence from the V_L-C_L junction in the Fab molecule were employed with some success. However, many aspects of linker design including "natural" versus "synthetic" have yet to be examined. In this report, we describe the production of several sFv constructs and the attempted production of Fv in the periplasm of *Escherichia coli* by expressing synthetic genes encoding these molecules. The effects of linker sequence and the orientation of the V_H and V_L domains on expression levels, secretion, and antigen binding properties were examined. Two types of linkers were used: (i) sequences derived solely from the elbow regions of the corresponding Fd or light chains and (ii) sequences incorporating the previously used GGGGS sequence (3, 4). The antibody we selected was Se155-4, specific for a trisaccharide epitope of *Salmonella* serogroup B *O*-polysaccharide (9). Previously, Se155-4 Fab has been produced in *E. coli* using synthetic dicistronic DNA and has been shown to be as active as mouse Fab in antigen binding and competitive immunoassays (10, 11).

MATERIALS AND METHODS

Enzymes, Oligomers, and General Techniques—Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs, GIBCO-BRL and Boehringer Mannheim. Deoxyribonucleotides were synthesized using an automatic DNA synthesizer model 380A (Applied Biosystems Inc.). Plasmid pB*tac*2 was purchased from Boehringer Mannheim. Goat anti-mouse λ antibody conjugated to alkaline phosphatase and anti-mouse λ/biotin conjugates were purchased from Caltag. Other reagents used in EIA were purchased from Kirkegaard and Perry Laboratories, GIBCO-BRL, or Bio-Rad. Mouse Fab, *E. coli*-produced Fab and Se155-4 IgG were

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[1] The abbreviations used are: V_L, light chain variable domain; BSA, bovine serum albumin; EIA, enzyme immunoassay; Fv, fragment containing V_L and V_H domains; λ, lambda light chain; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sFv, single-chain Fv; V_H, heavy chain variable domain.

obtained by affinity chromatography as described previously (11).

Construction of Fv Expression Plasmid—The plasmid pSal V_L was derived from pSal-L (10) and was joined at its 3′ *Sac*I terminus with the V_H gene at its 5′-*Eco*RI site by a synthetic 24-nucleotide spacer duplex (11). This spacer duplex contained a ribosomal binding site eight nucleotides from the ATG start codon of the *ompA* signal peptide preceding the V_H gene. The resultant gene construct is shown in Fig. 1*A*. Both variable domains are preceded by the *ompA* signal peptide allowing their secretion into the periplasm of *E. coli*. For expression studies, the *Eco*RI-*Hin*dIII cassette containing the cistronic genes was cloned into the corresponding sites of the pB*tac*2 plasmid and designated ptFv.

Construction of sFv Expression Plasmids—Four sFv genes in both orientations, V_L-V_H or V_H-V_L, carrying both elbow (el) and flexible (fl) linkers were constructed from plasmids carrying the V_L, V_H or the dicistronic Fab genes (10, 11). As in the Fv construct, suitable restriction fragments carrying the desired sequences were bridged by synthetic oligonucleotides. The organization of all four genes and the linker sequences incorporated into the four designs are shown in Fig. 1*B*. The 5′-end of each gene was preceded by the *ompA* secretory signal. The four constructs were cloned into the pB*tac*2 vector for the purpose of expression. The recombinant pB*tac*2-derived plasmids were designated ptsFvLH(el), ptsFvLH(fl), ptsFvHL(el), and ptsFvHL(fl).

Expression of Fv and sFv—The plasmid ptFv harboring the cistronic Fv gene was transformed into competent *E. coli* TG1 cells and grown in shake flasks at 30 °C in M-9 minimal medium supplemented with 0.4% casamino acids and 100 μg/ml of ampicillin. At 48 h, the cultures were induced with supplementary nutrients (12 g of tryptone, 24 g of yeast extract, 4 ml of glycerol/liter) and 2 mM isopropylthiogalactopyranoside. The cultures were harvested 24 h later and periplasmic extracts prepared as described earlier (11). Periplasmic extracts were checked for activity by indirect EIA and also analyzed by SDS-PAGE and Western blotting. SDS-PAGE (12.5% acrylamide) was performed using the buffer system described by Laemmli (12), and gels were stained with Coomassie Brilliant Blue. In Western blotting, proteins were transferred to Immobilon-P (Millipore) with 25 mM Tris-glycine, pH 8.2, containing 15% methanol as the transfer buffer. The detection reagent was goat anti-mouse λ/alkaline phosphatase. After extensive dialysis against 50 mM Tris-HCl, pH 8.0, buffer containing 0.15 M NaCl, the extracts were applied to an antigen-based affinity column as described earlier (11).

Plasmids harboring the sFv genes were transformed into *E. coli* TG1 cells. Cultures were grown as described above, and the single chain products were isolated from periplasmic extracts by affinity chromatography in a similar manner.

Quantitation of sFv Expression and Secretion—Cultures (100 ml) harboring ptsFvLH(el), ptsFvLH(fl), ptsFvHL(el), or ptsFvHL(fl) were grown and induced as described above. The periplasmic extracts were prepared from 50 ml of each culture. The two supernatants obtained by sucrose and shock treatment, respectively, were combined (total volume, 5 ml). The cells from the remaining 50 ml of each culture were suspended in 5 ml of water. Dilution series of cell suspensions and periplasmic fractions were analysed by SDS-PAGE/Western blotting and the amounts of single-chain product estimated by comparison with a dilution series of purified product.

sFv Cleavage—Purified V_L-V_H(el) sFv was incubated at 30 °C in PBS for up to 7 days at an antibody concentration of 300 μg/ml. To test for inhibition of cleavage by protease inhibitors, incubations were set up with each of the following inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, aprotinin (10 μg/ml), E-64 (1 mg/ml), and pepstatin (0.7 μg/ml). Aliquots were taken at appropriate intervals for analysis by SDS-PAGE, Western blotting, and EIA.

Enzyme Immunoassay—Indirect EIA was carried out using microtiter plates coated with BSA-*O*-polysaccharide conjugate[2] at a concentration of 10 μg/ml. Serial dilutions of antibody fragments were added to the plates, and bound materials were detected with an anti-mouse λ/biotin conjugate and streptavidin-horseradish peroxidase using 3,3′,5,5′-tetramethylbenzidine/H_2O_2 as substrate. Indirect EIA was also carried out with *Salmonella essen* lipopolysaccharide-coated plates, as previously described (10), using a goat anti-mouse λ chain antibody alkaline phosphatase conjugate.

Competition measurements were conducted using a direct EIA system (13). Protein was absorbed to plates, and the procedure was modified slightly in that the BSA blocking step was omitted. All incubations were done at 37 °C, and all buffers contained 0.05% Tween 20. The binding of biotinylated *O*-polysaccharide to antibody fragments in the wells was inhibited by various concentrations of *O*-polysaccharide (14). In addition, a competitive indirect EIA was developed for purposes of comparing the four sFvs and *E. coli*-produced Fab. In this assay microtiter plates were coated with a BSA-*O*-polysaccharide conjugate (as above). Antibody fragments were used at concentrations giving 70% of the maximum signal in indirect EIA with BSA-*O*-polysaccharide conjugate. The fragments were mixed with various concentrations of *O*-polysaccharide prior to addition to the wells. Bound antibody fragments were detected with an anti-mouse λ/biotin conjugate and streptavidin/horseradish peroxidase using TMB/H_2O_2 as the substrate.

Titration Microcalorimetry—Microcalorimetry was carried out on an OMEGA titration microcalorimeter from Microcal Inc. (Northampton, MA). This instrument has been described in detail by Wiseman *et al.* (15). Approximately 6–7 mg of protein in 1.3 ml of PBS, pH 7.0, was placed in the calorimeter cell and was stirred at 400 rpm at 25 °C. The sample was titrated with a 2 mM solution of the synthetic trisaccharide methyl 2-*O*-(α-D-galactopyranosyl)-3-*O*-(-3,6-dideoxy-*xylo*-α-D-hexopyranosyl)-α-D-mannopyranoside (the *Salmonella* serogroup B epitope) in PBS using 20 aliquots of 5 μl added at 3-min intervals from a 100-μl syringe. The reference cell was filled with water, and the instrument was calibrated by standard electrical pulses. The non-linear least squares analysis was performed as described elsewhere (9).

RESULTS

Expression of Fv Genes—The previously successful strategy of secreting the λ and Fd chains of Se155-4 Fab to the *E. coli* periplasm (10, 11) where correctly folded and active molecules were formed was applied to the V_L and V_H domains of Fv. Significant amounts of a protein of M_r 13,000 were detected in the periplasmic fractions of cultures harboring the ptFv plasmid by SDS-PAGE/Western blotting with anti-λ chain antibody, indicating V_L expression. However, no Fv product was detectable by affinity chromatography suggesting problems with V_H expression, V_H secretion, or a lack of V_H-V_L association in the periplasm.

Assembly of sFv Genes—Four sFv genes were constructed, two with V_L in the amino-terminal position and two with V_H in the amino-terminal position (Fig. 1*B*). Each orientation was constructed using flexible and elbow linkers. The linker designs incorporated sequences from the V_L-C_L elbow, the V_H-C_{H1} elbow, and the flexible sequence $(GGGGS)_2$ (Fig. 1*B*). An additional feature of the V_L-V_H elbow linker was the incorporation of a chemical cleavage site. This linker was composed of 12 amino acids from the NH_2 terminus of the C_L domain (ending with Ser^{125}) followed by Asn-Gly, a dipeptide sequence which is sensitive to hydroxylamine cleavage. The objective was to produce a "pro-protein" that could be cleaved in the folded state. The V_H-V_L elbow linker version of the molecule did not contain the hydroxylamine cleavage site and was composed solely of a 16 amino acid sequence from the C_{H1} domain (Fig. 1*B*).

The sequence $(GGGGS)_2$ was used to create flexible linker versions of each orientation (Fig. 1*B*). The location of suitable restriction sites dictated the incorporation of six constant domain amino acids into the V_L-V_H design and 5 constant domain residues into the V_H-V_L design. Hydroxylamine-cleavage sites were not included in either construct.

Expression and Secretion Levels—It was observed that the levels of secreted product with the V_L-V_H(el) construct were approximately 20-fold higher when the gene was under the control of the *tac* promoter, as compared to the *lac* promoter (data not shown). The effect of domain orientation and linker sequence on expression and secretion was studied using the *tac* series of plasmids. Periplasmic extracts from cells harboring the V_L-V_H constructs were shown by SDS-PAGE/Western blotting to contain significantly more single-chain product than periplasmic extracts from cells harboring the V_H-V_L

[2] E. Altman, unpublished results.

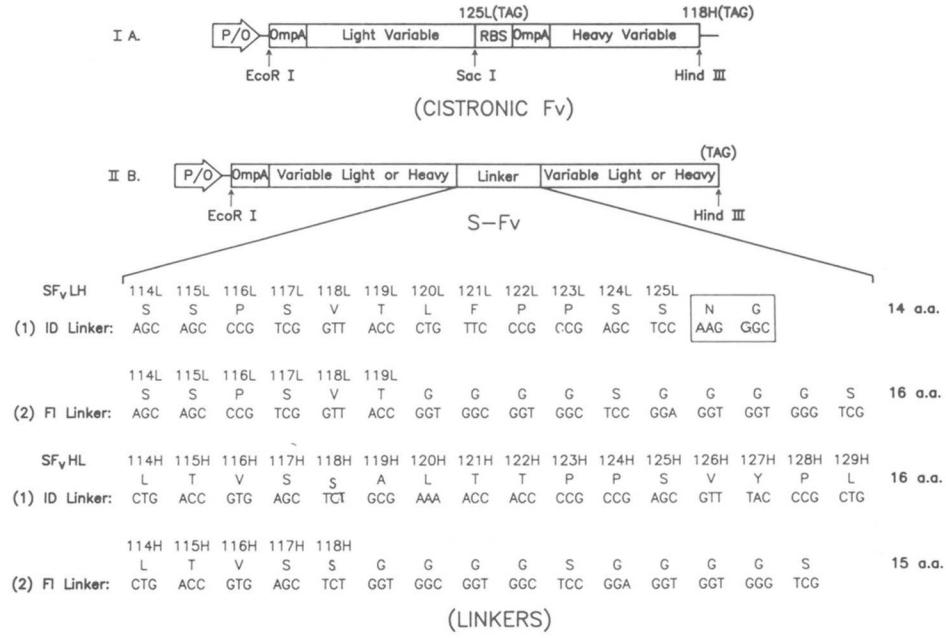

FIG. 1. **Fv and sFv vector constructs.** *A*, Fv vector construction for the expression of V_L and V_H as secreted products. *B*, generalized vector construction for the expression of sFvs as secreted products, and the linker sequences used in the four sFv constructs.

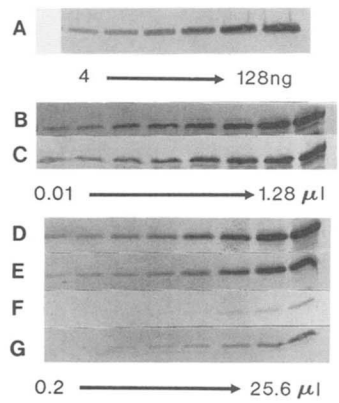

FIG. 2. **Western blotting of dilution series of purified sFv (*A*), V_L-V_H(el) cell suspension (*B*), V_H-V_L(el) cell suspension (*C*), V_L-V_H(el) periplasmic extract (*D*), V_L-V_H(fl) periplasmic extract (*E*), V_H-V_L(el) periplasmic extract (*F*) and V_H-V_L(fl) periplasmic extract (*G*).** Cell suspension and periplasm volumes refer to volumes of original culture corresponding to each dilution series. The cell suspensions of the flexible linker constructs gave results similar to those obtained for the elbow linker constructs. The dilutions of the cell suspensions and periplasmic extracts were added to an equal volume of SDS sample buffer containing 5% β-mercaptoethanol and boiled for 5 min prior to application to the gels.

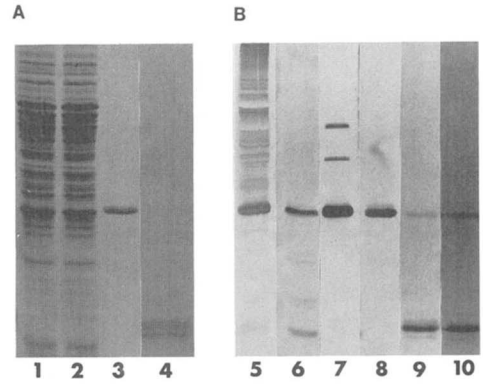

FIG. 3. **Analysis of V_L-V_H(el) sFv purification and cleavage.** *A*, SDS-PAGE. *1*, periplasmic extract; *2*, flowthrough from antigen column; *3*, purified sFv; *4*, cleaved sFv. All samples were prepared with sample buffer containing 5% β-mercaptoethanol. *B*, Western blot analysis. *5* and *6*, periplasmic extract; *7* and *8*, purified sFv; *9* and *10*, cleaved sFv. Samples in the *odd-numbered lanes* were prepared under non-reducing conditions while those in *even-numbered lanes* were reduced.

plasmids (Fig. 2). Only the sFv regions of the Western blots are shown in Fig. 2, but a complete and representative Western blot of a periplasmic extract is shown in *lane 6* of Fig. 3. Levels of secreted product for the V_L-V_H(el), V_L-V_H(fl), V_H-V_L(el), and V_H-V_L(fl) constructs were estimated to be 2400, 2400, 80, and 160 μg/liter of culture, respectively. With all constructs, a relatively small fraction of the total product was secreted. The levels of expression were similar in each instance, approximately 50 mg/liter.

Single-chain Fv Purification—The products of the sFv genes were isolated from periplasmic extracts in a single step by affinity chromatography (Fig. 3). This also provided a more accurate means of confirming the secretion levels estimated by SDS-PAGE/Western blotting (Fig. 2). The values obtained for each construct by the two procedures were in excellent agreement. The affinity-purified products gave single Coomassie-staining bands on both non-reducing and reducing SDS-PAGE gels (Fig. 3). Two minor, higher molecular weight species were detected by Western blotting (Fig. 3). These may be the products that are cross-linked through the free cysteine at position 94 of the light chain, since these bands disappeared on reduction. NH_2-terminal amino acid sequence analysis of the V_L-V_H(el) product showed correct processing of the leader sequence and confirmed the NH_2-terminal sequence up to residue 20.

Single-chain Fv Cleavage—Use of the Asn-Gly site for the specific cleavage of the linker region by hydroxylamine was made unnecessary by the observation that there was spontaneous cleavage of the V_L-V_H(el) linker upon prolonged storage at 4 °C. Incubation of the sFv at 30 °C gave virtually complete cleavage after 4 days (Fig. 3). Complete binding to the antigen column of the cleaved product at a protein concentration of 4.5 mg/ml indicated that it existed in a fully active form under these conditions. NH_2-terminal amino acid sequence analysis indicated that peptide cleavage had occurred between Ser^{124} and Ser^{125}. The cleavage could be completely inhibited by 1 mM EDTA suggesting that the hydrolysis was mediated by a metalloprotease. Hydrolysis was unaffected by the other pro-

tease inhibitors that were tested. Since Fv itself could not be obtained (see above), this cleaved sFv was used instead for immunological and microcalorimetric comparisons with intact sFv.

Indirect EIA—The antigen-binding activities of the sFv products were compared by indirect EIA with Fab produced in *E. coli* (Fig. 4). Similar patterns were obtained with lipopolysaccharide or BSA-*O*-polysaccharide as the antigen, but the results were more consistent and differences more pronounced with the BSA conjugate and the anti-mouse λ-biotin detection system. If the amount of antibody required to give 50% maximum activity is used as an indication of affinity, the assay showed that the sFvs and the Fab were generally quite similar on a mass basis. The V_H-V_L(fl) construct consistently displayed the highest binding, which was approximately 10-fold higher than the least active V_L-V_H(fl) construct. The activity profiles of intact and cleaved V_L-V_H(el) were similar.

Competitive EIA—It was observed that while a blocking step with bovine serum albumin did not significantly affect the signal obtained with mouse Fab in direct EIA, it reduced that obtained with *E. coli*-produced Fab and sFv by over 90%. Fortunately, a blocking step was found not to be necessary in this assay. The underlying cause of this difference in response was not investigated.

Bacterially produced Se155-4 Fab was compared with the V_L-V_H(el) sFv using this assay (Fig. 5). The concentration of *O*-polysaccharide antigen required to inhibit binding of biotin-labeled *O*-polysaccharide was identical for both preparations, indicating similar affinity for antigen.

The competitive indirect assay was the method of choice for comparing all four sFv constructs because the V_H-V_L constructs could not be detected in the direct assay. In this system, affinity for antigen is inversely proportional to inhibitor concentration at 50% inhibition (13). Results obtained with this assay (Fig. 6) were in good agreement with those described above for indirect EIA which show that the V_H-V_L(fl) has a 10-fold high affinity compared to V_L-V_H(fl). The other constructs showed similar activities. The cleaved form of the V_L-V_H(fl) had an affinity similar to the intact molecule.

Microcalorimetry—The thermodynamics of hapten-binding

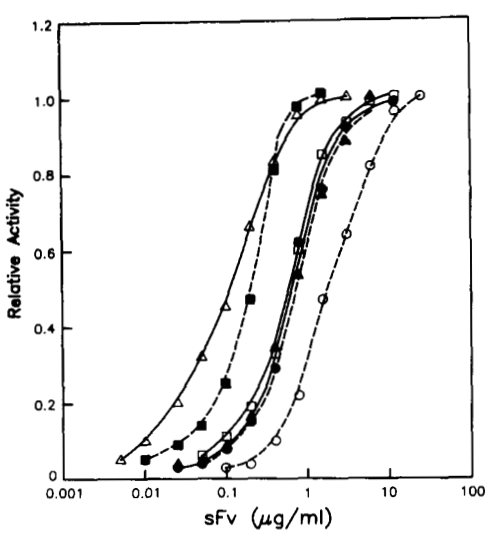

FIG. 4. **Indirect EIA of *E. coli*-produced Fab (■---■), V_L-V_H(el) sFv (●——●), V_L-V_H(fl) sFv (○---○), V_H-V_L(el) sFv (▲---▲), V_H-V_L(fl) sFv (△——△), and cleaved V_L-V_H(el) sFv (□——□) using microtiter plates coated with BSA-*O*-chain and detection with goat anti-mouse λ/biotin conjugate and streptavidin/horseradish peroxidase.**

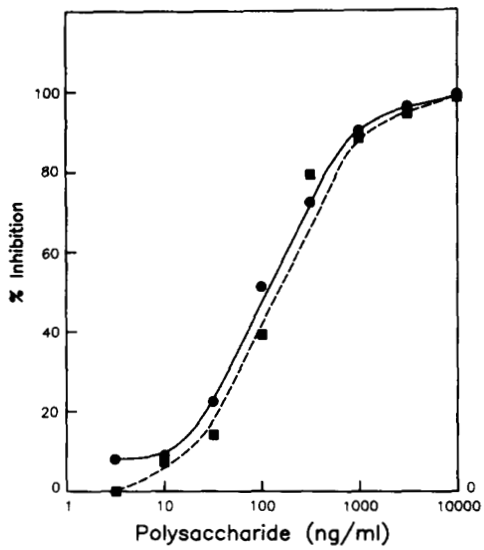

FIG. 5. **Competitive direct EIA of *E. coli*-produced Fab (■---■) and single-chain V_L-V_H(el) (●——●), using *O*-polysaccharide as inhibitor of the binding of *O*-polysaccharide/biotin conjugate.**

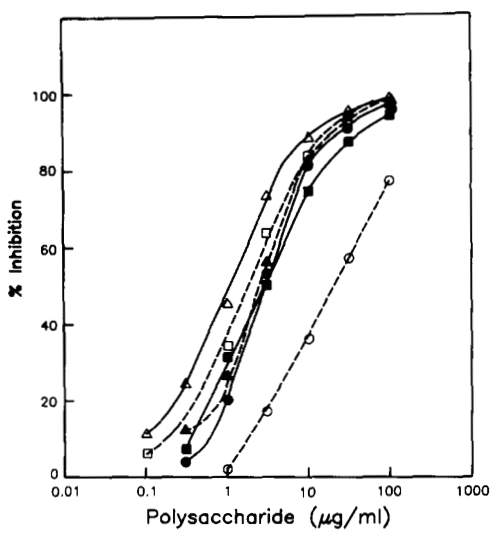

FIG. 6. **Competitive indirect EIA of *E. coli*-produced Fab (■——■), V_L-V_H(el) sFv (●——●), V_L-V_H(fl) sFv (○---○), V_H-V_L(el) sFv (▲---▲), V_H-V_L(fl) sFv (△——△), and cleaved V_L-V_H(el) sFv (□---□), using BSA-*O*-polysaccharide-coated plates, *O*-polysaccharide inhibitor, and fixed amounts of sFv, detected with goat anti-mouse λ/biotin and streptavidin/horseradish peroxidase.**

TABLE I

Comparison of the binding properties of IgG, E. coli-produced Fab, sFv, and cleaved sFv by titration microcalorimetry

	K	$\Delta G°$	$\Delta H°$	$-T\Delta S°$	$\Delta S°$
	$M^{-1} \times 10^5$		*kJ mol*$^{-1}$		*J mol*$^{-1}$ *K*$^{-1}$
Mouse IgG	2.1 ± 0.3	−31 ± 1	−21 ± 2	−10 ± 1	+34 ± 3
E. coli Fab	1.3 ± 0.5	−29 ± 1	−19 ± 1	−10 ± 2	+34 + 7
V_L-V_H(el)	1.3 ± 0.5	−29 ± 1	−24 ± 1	−4 ± 2	+18 ± 5
Cleaved V_L-V_H(el)	0.6 ± 0.5	−27 ± 2	−26 ± 6	−1 ± 8	+4 ± 25

to mouse IgG, *E. coli*-produced Fab, single-chain V_L-V_H(el), and cleaved single-chain V_L-V_H(el) were determined by microcalorimetry (Table I). Association constants were also determined from the same data and showed little significant difference for the four species. There was a corresponding agreement in the enthalpy of association. The results for the

cleaved Fv were less accurate than other measurements since the experiment was performed at a somewhat lower protein concentration (Table I). The observed association constants in the order of 10^5 M^{-1} lay in the range typical of antibody-carbohydrate binding. As reported previously (9), the binding of the trisaccharide epitope is mostly enthalpy driven but with a significant contribution from the entropy term ($-T\Delta S°$).

DISCUSSION

Single-chain antibody fragments are one of the most novel developments in the rapidly evolving area of antibody engineering (1, 16). Although bacterial leader sequences have been used to target Fab (17) and Fv (18) products to the periplasm, most sFvs so far constructed were expressed as inclusion bodies in *E. coli* (3, 4). While inclusion body isolation followed by denaturation and refolding steps can give acceptable yields of active sFv, the procedure is tedious and plagued by solubility problems. Our present study reports on four aspects of sFv production: (i) the targeting of sFv to the *E. coli* periplasm, (ii) the effect of domain orientation on secretion, (iii) the feasibility of using linker sequences derived solely or partially from the elbow regions of the corresponding Fab molecule, and (iv) the effect of linker sequence and domain orientation on antigen binding properties. We also observed the serendipitous proteolytic cleavage of our sFv within the peptide linker sequence, thereby yielding an active Fv.

A major observation reported in the present study was that the uniformly high expression levels of products from all four constructs made with the Se155-4 V_L and V_H domains did not translate into correspondingly high yields of secreted protein. Less than 5% of the expressed sFv was secreted, and this secretion was significantly dependent on domain orientation in the construct. Nevertheless, there are obvious advantages in avoiding the harsh denaturing conditions required for purification of sFvs from inclusion bodies, and the yields of secreted products, while acceptable for many purposes, could probably be improved by changing factors such as culture conditions, bacterial strain, or leader sequence.

With a V_H-V_L orientation, the secreted yield was 20-fold less than that obtained with a V_L-V_H orientation. Although some contribution to this effect by minor differences in linker sequence is possible, the major factor of domain orientation is thought to be primarily responsible. While there are many examples of heterologous protein secretion to the *E. coli* periplasm, the molecular mechanisms involved in the process are not completely understood. Attachment of a signal sequence does not, in itself, always lead to secretion (19), implying that information contained in the mature protein sequence is also important (20). It has been suggested that entry to the prokaryotic secretory pathway is contingent on a protein assuming a soluble and suitably folded conformation (21). Domain orientation might significantly affect the degree to which sFv molecules meet such a requirement. Some findings relating to heavy chain secretion in mammalian cells may also apply in bacterial systems. Polypeptide chain-binding proteins implicated in protein folding can be grouped into families that span the prokaryote/eukaryote boundary (31). In mammalian cells, secretion of immunoglobulin molecules is mediated by the light chain and is impeded when light chain is limiting (22). Also, while free light chain is readily secreted (23, 24) free heavy chain is generally not secreted (25, 26, 27, 28). In sFv molecules, V_H at the NH_2-terminal position may limit secretion because of its folding taking place in the absence of V_L, resulting in inclusion body formation.

In the present study, the use of elbow region sequences as linkers in sFvs resulted in molecules that were indistinguishable from the corresponding Fab molecule with respect to antigen binding thermodynamics as measured by EIA (Figs. 4–6) and titration microcalorimetry (Table I). This contrasts with the report by Condra *et al.* (5) in which an sFv containing an elbow linker had less than 10% of the activity of the Fab molecule. However, these authors presented evidence suggesting that the elbow sequence they used was too short. If suitable restriction sites are present, the use of elbow sequences can provide a straight-forward means of generating sFv genes from the Fab genes. In addition, a natural linker may have lower immunogenicity for *in vivo* applications. Here we also incorporated a chemical cleavage site in one linker sequence so that the sFv product could serve as a "pro-protein" for the Fv product. It was found that chemical cleavage was unnecessary, the sFv being successfully cleaved by an endogenous enzyme to yield a fully functional Fv. Although the enzymic nature of the cleavage was established, the origin of the enzyme and its fortuitous copurification with the sFv were not investigated. This enzymic cleavage was particularly useful for characterization purposes as we were unable to isolate an Fv molecule by expression of dicistronic DNA constructs, possibly because of poor V_H expression or secretion. Field *et al.* (29) found that V_L was expressed far better than V_H for an anti-lysozyme F_v, also suggesting unfavorable V_H properties.

The EIA data using both indirect and direct assay formats suggested that the various constructs had comparable binding constants with the 50% inhibition points all falling within an order of magnitude. For the Se155-4 constructs, linker attachment through Gly as in the V_L-V_H(el) sFv gave a little better affinity than attachment through Ser as in the V_L-V_H(fl), and the V_H-V_L(fl) was better than either of these. Longer range effects of other sequence differences in the linkers may also be important. These differences may be related to the effects that changes in the NH_2-terminal residues of the V_H can have on affinity for antigen, as shown with antibodies and sFv specific for digoxin (3). The effects may be explained in terms of a H-bond network connecting the NH_2-terminal residues to residues in the binding site (30).

The similarity of Fab and sFv binding constants was confirmed by titration microcalorimetry for sFv V_L-V_H(el). Where quantities of purified protein permit, titration microcalorimetry provides an accurate and powerful method for the elaboration of a complete thermodynamic description of ligand-protein interaction. In the present study, the binding thermodynamics for the trisaccharide hapten to mouse IgG and the three cloned products *E. coli* Fab, single-chain V_L-V_H(el), and cleaved single-chain V_L-V_H(el), were shown to be indistinguishable. The favorable contribution of entropy to the free energy of binding at 25 °C, observed with this mouse IgG, and also consistently observed for the bacterially expressed fragments, is an unusual trait in protein-carbohydrate interactions. It is interesting to note that there is no significant change in the thermodynamic functions between the sFv and the cleaved sFv. Presumably, the concentration of the protein in the microcalorimeter (approximately 50 μM) is high enough that there is a very low concentration of the dissociated form of the Fv. Alternatively the binding of the hapten might increase the association of the V_L and V_H chains (2).

In conclusion, the experiments reported here show that successful design of genes for sFv products with good affinity and secretion levels will involve a balance of two opposing factors. These are the benefits of placing the V_L domain in the NH_2-terminal position to improve secretion, and the negative effects that attachment of the linker sequence to the NH_2-terminal residue of the V_H domain can have on the affinity of the sFv. Hence, for any parent antibody, optimal

design of the sFv for good affinity and secretion level cannot be done to a formula, but instead will require evaluation of the two factors by experiments of the kind we have conducted with Se155-4.

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