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 inclusion bodies. With V_L in the NH2-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 bacterial 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 and V_H) at the amino termini of the heavy and light chains. For in vivo diagnostic and immunotherapeutic 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 NH2 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)_2 have been used, but in one report (5) linkers incorporating an interdomain sequence from the V_L-C_V_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 automated DNA synthesizer model 380A (Applied Biosystems Inc.). Plasmid pPtac2 was purchased from Boehringer Mannheim. Goat anti-mouse λ antibody conjugated to alkaline phosphatase and anti-mouse λ/β 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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* The abbreviations used are: V_L, light chain variable domain; BSA, bovine serum albumin; ELA, 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 Plasmids—The plasmid pSal Vl was derived from pSal-L (10) and was joined at its 3'-SacI site with the Vl gene at its 5'-EcoRI 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 gene. The resultant gene construct is shown in Fig. 1A. Both variable domains are preceded by the ompA signal peptide allowing their secretion into the periplasm of E. coli. For expression studies, the EcoRI-HindIII cassette containing the atomic genes was cloned into the corresponding sites of the pBtac2 plasmid to yield the pBtac2-derivatives designated pFv.

Construction of sFv Expression Plasmids—Four sFv genes in both orientations, Vl-VH or VH-Vl, carrying both elbow (el) and flexible (fl) linkers were constructed from plasmids carrying the Vl, VH 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. 1B. The 5'-end of each gene was preceded by the ompA secretory signal. The four constructs were cloned into the pBta2 vector for the purpose of expression. The recombinant pBtac2-derived plasmids were designated pFsVH(LH)el, pFsVH(LH)fl, pFsVLH(el), and pFsVLH(fl).

Expression of Fv and sFv—The plasmid pFv 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 0.004 M asparagin. 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 isopropylthio-
d-galactopyranoside. 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 X/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 pFsVH(LH)el, pFsVH(LH)fl, or pFsVLH(fl) were cultivated 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 Vl-Vh (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 microtitre plates coated with BSA-O-polysaccharide conjugate 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/histone conjugate and streptavidin-horseradish peroxidase using 3,3',5,5'-tetramethylenediamine/H2O2 as substrate. Indirect EIA was developed with Salmonella serogroup B 0-polysaccharide-coated plates, as previously described (10), using a goat anti-mouse λ chain antibody alkaline phosphatase conjugate.

The sequence (GGGGS)2 was used to create flexible linker versions of each orientation (Fig. 1B). The location of suitable restriction sites dictated the incorporation of six constant domain amino acids into the Vl domain, and two with VH in the amino-terminal position (Fig. 1B). Each orientation was constructed using flexible and elbow linkers. The linker derived incorporated sequences from the Vl-Cv, CH1, elbow, and the flexible sequence (GGGGS)2 (Fig. 1B). An additional feature of the Vl-VH elbow linker was the incorporation of a chemical cleavage site. This linker was composed of 12 amino acids from the NH2-terminus of the Cv domain (ending with Ser25) followed by Asn-Gly, a dipetide sequence which is sensitive to hydroxylamine cleavage. The objective was to produce a "pro-protein" that could be cleaved in the folded state. The Vl-VH 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 Cv domain (Fig. 1B).

The sequence (GGGGS)2 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 Scil54-Fab to the E. coli periplasm (10, 11) where correctly folded and active molecules were formed was applied to the Vl and VH domains of Fv.

Significant amounts of a protein of Mr 13,000 were detected in the periplasmic fractions of cultures harboring the pFv plasmid by SDS-PAGE/Western blotting with anti-λ chain antibody, indicating Vl expression. However, no Fv product was detectable by affinity chromatography suggesting problems with VH expression, Vl secretion, or a lack of Vl-VH association in the periplasm.

Assembly of sFv Genes—Four sFv genes were constructed, two with Vl in the amino-terminal position and two with VH in the amino-terminal position (Fig. 1B). Each orientation was constructed using flexible and elbow linkers. The linker derived incorporated sequences from the Vl-Cv, elbow, CH1, and flexible sequence (GGGGS)2 (Fig. 1B). An additional feature of the Vl-VH elbow linker was the incorporation of a chemical cleavage site. This linker was composed of 12 amino acids from the NH2 terminus of the Cv domain (ending with Ser25) followed by Asn-Gly, a dipetide sequence which is sensitive to hydroxylamine cleavage. The objective was to produce a "pro-protein" that could be cleaved in the folded state. The Vl-VH 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 Cv domain (Fig. 1B).

The sequence (GGGGS)2 was used to create flexible linker versions of each orientation (Fig. 1B). The location of suitable restriction sites dictated the incorporation of six constant domain amino acids into the Vl domain and five constant domain residues into the VH domain. Hydroxylamine-cleavage sites were not included in either construct.

Expression and Secretion Levels—It was observed that the levels of secreted protein with the Vl-VH (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 Vl-VH constructs were shown by SDS-PAGE/Western blotting to contain significantly more single-chain product than periplasmic extracts from cells harboring the Vl-VH,

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* E. Altman, unpublished results.
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\textsubscript{i} - V\textsubscript{H} constructs were estimated to be 2400, 2400, 80, and 160 \mu g/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. N\textsubscript{H}\textsubscript{2}-terminal amino acid sequence analysis of the V\textsubscript{i} - V\textsubscript{H} product showed correct processing of the leader sequence and confirmed the NH\textsubscript{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\textsubscript{i} - V\textsubscript{H} 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. N\textsubscript{H}\textsubscript{2}-terminal amino acid sequence analysis indicated that peptide cleavage had occurred between Ser\textsuperscript{125} and Ser\textsuperscript{126}. 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-
Anti-Salmonella Single-chain Fvs

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 VH-VL(fl) construct consistently displayed the highest binding, which was approximately 10-fold higher than the least active Vh-Vl(fl) construct. The activity profiles of intact and cleaved Vl·Vh(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 Vl·Vh(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 VH-VL 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 Vh·Vl(fl) has a 10-fold high affinity compared to Vl·Vh(fl). The other constructs showed similar activities. The cleaved form of the Vl·Vh(fl) had an affinity similar to the intact molecule.

Microcalorimetry—The thermodynamics of hapten-binding to mouse IgG, E. coli-produced Fab, single-chain Vl·Vh(el), and cleaved single-chain Vl·Vh(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

Fig. 4. Indirect EIA of E. coli-produced Fab (■—■), Vl·Vh(el) sFv (O—O), and cleaved 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 Vl·Vh(el) (O—O), using O-polysaccharide as inhibitor of the binding of O-polysaccharide/ biotin conjugate.

Fig. 6. Competitive indirect EIA of E. coli-produced Fab (■—■), Vl·Vh(el) sFv (O—O), Vh·Vl(fl) sFv (△—△), Vl·Vh(el) sFv (■—■), Vh·Vl(fl) sFv (△—△), and cleaved Vl·Vh(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

|          | K (M⁻¹) | ΔG° (kJ mol⁻¹) | ΔH° (kJ mol⁻¹) | ΔS° (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 |
| Vl·Vh(el) | 1.3 ± 0.5 | -29 ± 1 | -24 ± 1 | -4 ± 2 | +18 ± 5 |
| Cleaved Vl·Vh(el) | 0.6 ± 0.5 | -27 ± 2 | -26 ± 6 | -1 ± 8 | +4 ± 25 |

Comparison of the binding properties of IgG, E. coli-produced Fab, sFv, and cleaved sFv by titration microcalorimetry.
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 $10^5 \text{M}^{-1}$ 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 ($-T \Delta S^\circ$).

**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 VL and VH 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 VH-VL orientation, the secreted yield was 20-fold less than that obtained with a VL-VH 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, thereby providing 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 VL-VH(el), and cleaved single-chain VH-VL(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 VH and VL 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 VH 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 VH domain can have on the affinity of the sFv. Hence, for any parent antibody, optimal
design of the scFv 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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