# Thermal stabilization of a single-chain Fv antibody fragment by introduction of a disulphide bond

N. Martin Young[a,*], C. Roger MacKenzie[a], Saran A. Narang[a], Raymond P. Oomen[a,**], John E. Baenziger[b]

[a]*Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, Ont. K1A 0R6, Canada*
[b]*Department of Biochemistry, University of Ottawa, Ottawa, Ont. K1H 8M5, Canada*

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**Abstract A disulphide bond was introduced into a single-chain Fv form of the anticarbohydrate antibody, Se155–4 by replacing Ala-L57 of the light chain and Asp-H106 of the heavy chain with cysteines, by site-directed mutagenesis. To maintain the salt-bridge from the latter residue to Arg-H98, Tyr-107 was also altered to Asp. The resulting ds-scFv was shown to retain full antigen-binding activity, by enzyme immunoassay and surface plasmon resonance analysis of binding kinetics. Compared with the parent scFv, the disulphide bonded form was shown to have enhanced thermal stability, by Fourier transform IR spectroscopy. The $T_m$ was raised from 60°C to 69°C. The ds-scFv form thus combines the stable monomeric form of the disulphide form with the expression advantages of the scFv.**

*Key words:* Antibody engineering; Fourier transform IR spectroscopy; Single-chain Fv; Thermal stability

## 1. Introduction

The antigen-binding site of an antibody is formed from the variable domains of its two chains. These associate through weak non-covalent interactions and their dimerization is aided by the positioning provided by the neighbouring constant domains in the Fab, which associate both non-covalently and covalently through a disulphide bond. Recombinant forms of the $V_L$-$V_H$ heterodimer, or Fv, can be expressed in *Escherichia coli* from cistronic genes [1], but in many instances these have shown poor $V_L$-$V_H$ association.

To improve Fv stability, two strategies have been developed. In the more common one, the two domains are assembled into a single polypeptide chain through a linker segment typically around 15 residues long, forming a single-chain Fv or scFv [1]. The second approach is to introduce a disulphide bond between the two domains, forming a ds-Fv. The positions of the two Cys replacements are chosen from the consideration of antibody crystal structures. The first design of this type by Glockshuber et al. [2] involved residues in the binding-site loops and is, therefore, not a general solution. Pastan and coworkers [3–5] have recently proposed two general disulphide designs, which are pseudo-symmetrically related.

We have identified another site for introduction of a disulphide bond in the antibody Se155–4, an $IgG_1\lambda_1$ anticarbohydrate antibody specific for the *Salmonella* serotype B polysaccharide [6] whose Fab and scFv structures have been determined at high resolution [7,8]. The site is intermediate between those of the above designs. We have incorporated the disulphide bond into an scFv version of the antibody and here show that this double strategy gives a fully active Fv with considerably better stability.

## 2. Materials and methods

### 2.1. Design of ds-scFv and its gene

The crystal structure of the Se155–4 Fab [7] was analysed using the Quanta software package (Polygen). The three mutations chosen were Ala-L57 to Cys, Asp-H106 to Cys and Tyr-H107 to Asp (sequential numbering; the Kabat equivalents are L55, H101 and H102). These mutations were introduced into clone 3B1, a Se155–4 mutant with an Ile-H77-Thr substitution that results in higher yields of functional scFv in *E. coli* [9]. This was done by inserting oligonucleotides between suitable restriction sites. All DNA manipulations were carried out by standard procedures [10].

### 2.2. Expression and purification of antibody fragments

The scFv and Fab were purified from periplasmic extracts as previously described [9,11]. Inclusion bodies containing ds-scFv were harvested from the bacterial cells and the antibody fragment was extracted, refolded and oxidized by the procedure of Buchner et al. [12], except that the oxidation was carried out at 4°C for 3 days. The products were concentrated and the ds-scFv was purified by affinity chromatography on an antigen matrix [6]. The monomer forms of both scFv and ds-scFv were purified by size-exclusion HPLC on Superdex 75 (Pharmacia Biotech). For cleavage of the linker, samples in 0.1 M Tris buffer, pH 8.0, were incubated with 1:500 subtilisin at 37°C; reactions were stopped by addition of 0.1 M phenylmethylsulphonylfluoride in dimethylsulphoxide.

### 2.3. Immunological analyses

Enzyme immunoassays (EIAs) with antigen-coated plates were performed as previously described [13]. Interaction analysis by surface plasmon resonance [14] was carried out with a BIAcore instrument (Pharmacia Biosensor, Piscataway, NJ) and the kinetic parameters were determined using the BIAevaluation 2.0 software. The antigen used was a conjugate of the *Salmonella* O-chain and bovine serum albumin and it was attached to the dextran-gold surface, using the amine coupling kit supplied by the manufacturer, in 10 mM Na acetate, pH 4.5, at concentrations and contact times that yielded about 200 RU of immobilized material. 1 RU corresponds to an immobilized protein concentration of 1 pg/mm$^2$ [15]. All measurements were performed at ambient temperature in 10 mM HEPES, pH 7.4, 100 mM NaCl, 3.3 mM EDTA at a flow rate of 5 μl/min and protein concentrations between 1.8 and 4.6 μM. Surfaces were regenerated with 10 mM HCl.

*Corresponding author. Fax: (1) (613) 941 1327.

***Present address:* Connaught Center for Biotechnology Research, 1755 Steeles Avenue West, Willowdale, Ont. M2R 3T4, Canada.

*Abbreviations:*: EIA, enzyme immunoassay; FTIR, Fourier transform IR spectroscopy; scFv and ds-scFv, single-chain Fv and disulphide-stabilized scFv.


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*2.4. Thermal denaturation analysis by FTIR*

FTIR spectra were acquired on an FTS-40 spectrometer, equipped with an MCT detector (BioRad, Cambridge MA) and continually purged with dry air (dew point −100°C) from an air-dryer (Balston, Haverhill, MA). Samples were exchanged into $^2H_2O$ for approximately 24 h, then placed between two $CaF_2$ windows separated by a 12-$\mu$m Teflon spacer. The temperature of the sample was regulated by circulating water through a thermostatically controlled jacket surrounding the $CaF_2$ windows (Harrick, Ossining, NY). Spectra were recorded at 2 $cm^{-1}$ resolution and represent the average of 256 scans.

## 3. Results

*3.1. Design and production of the ds-scFv*

The positions for mutation to Cys to create an interdomain disulphide bond in the Fv were identified within the Se155–4 structure [7] by visual inspection of residue $C_\alpha$-$C_\beta$ bond vectors and measurement of $C_\alpha$-$C_\alpha$ distances using the Quanta software package. The $V_L$ residue was Ala-L57 and the $V_H$ residue was Asp-H106, and their relationship is shown in Fig. 1. The distance between the two $C_\alpha$ atoms was 5.9 Å. In many antibodies, the residue Asp-H106 is a partner in a highly-conserved salt-bridge with Arg-H98. However, previous mutagenesis experiments [15] had shown that replacement of the next residue, Tyr-H107, with Asp gave an Fab with full antigen-binding activity. In order to retain an acidic group in this location as a potential salt-bridge partner for Arg-H98, this replacement was made as well. The three mutations were introduced into an scFv gene which had previously been found to give excellent expression into the periplasm of *E. coli* [9]. However, the mutant form gave a poorer yield of soluble protein and the ds-scFv was, therefore, prepared from the inclusion bodies by denaturation, refolding and oxidation, by a standard method [11]. The overall yield of ds-scFv after purification by affinity chromatography was 25 mg/l of culture.

*3.2. Characterization of the ds-scFv*

To demonstrate formation of the interdomain disulphide bond, a mild proteolytic cleavage was used to sever the linker peptide. Preliminary experiments with the parent scFv showed that treatment with subtilisin at 1:500 for 2 h cleaved the linker without degrading the domains (Fig. 2, lanes 2 and 7). When ds-scfv molecules were treated in this way, the apparent molecular weight on SDS-PAGE was little changed (Fig. 2, lane 4) but after the product was reduced, fragments of the size of $V_L$ and $V_H$ domains were seen (Fig. 2, lane 9). The disulphide bond was, therefore, fully formed in the product.

The reactivity of the ds-scFv with antigen was first assessed by EIA. The parent scFv and the ds-scFv showed very similar

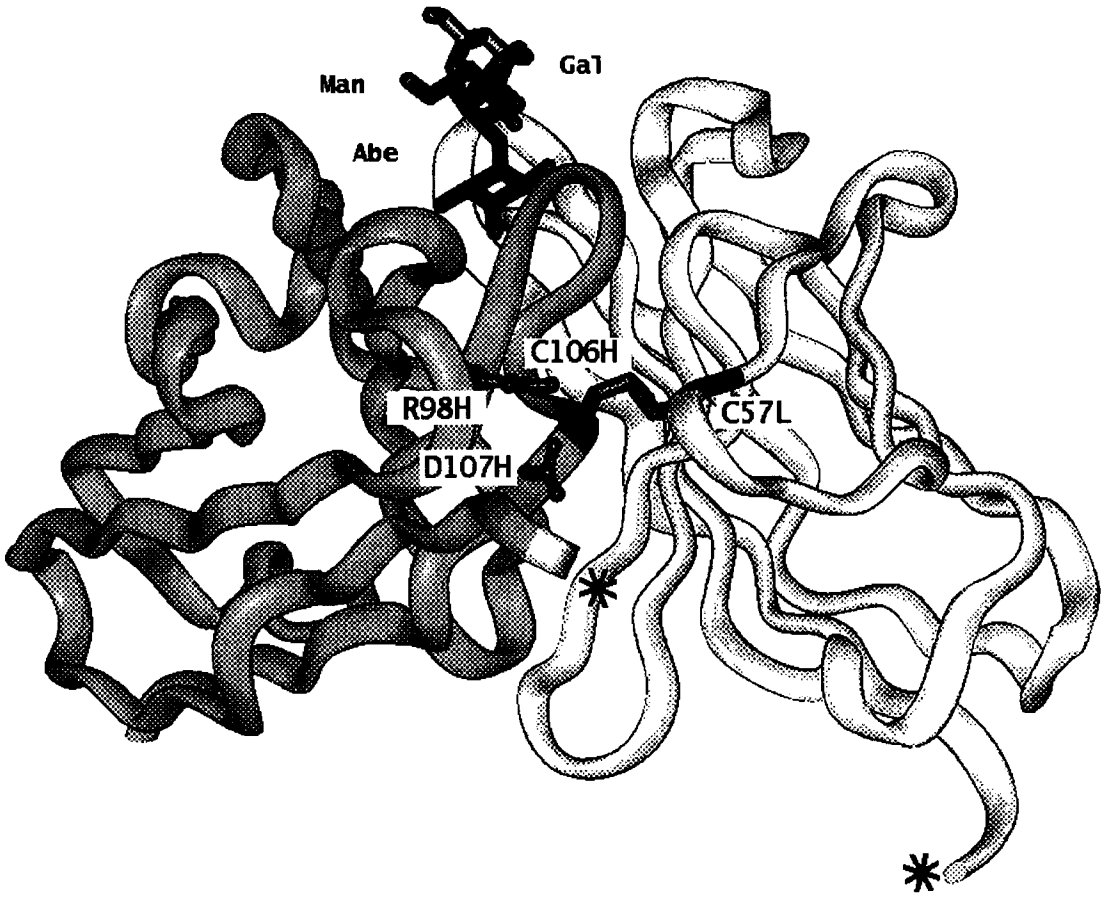


Fig. 1. A ribbon diagram of the Se155–4 scFv showing the location of the introduced disulphide bond. The light and heavy chains are lighter and darker shades of grey, respectively, and the trisaccharide antigen is shown bound in the combining site. The introduced cysteine residues at position 57L and 106H are labeled C57L and C106H, respectively, and the disulphide joining them has been modeled. The side chain of the other mutated residue in this construct (Tyr → Asp) and the conserved arginine with which it may form a salt bridge are also shown (D107H and R98H, respectively). The large asterisks denote the starting and ending points of the linker connecting the VL and VH domains. This figure is based on Brookhaven Protein Data Bank file 1mfa and was prepared with InsightII software from Biosym.

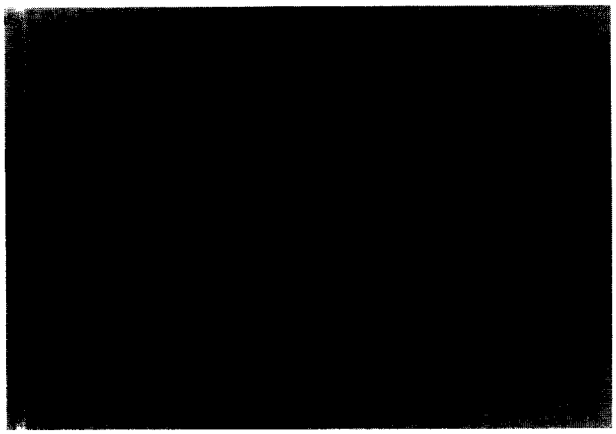


Fig. 2. SDS-PAGE of Se155–4 scFv and ds-scFv. Lanes 1–4 are samples without reduction; lanes 6–9 are reduced samples. Lanes 1 and 6, scFv without subtilisin treatment; lanes 2 and 7, scFv after 1:500 subtilisin treatment for 3 h; lanes 3 and 8, ds-scFv without subtilisin treatment; lanes 4 and 9, ds-scFv after 1:500 subtilisin treatment for 3 h; lane 5, standards, from bottom, lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin.

activity (Fig. 3). More detailed analysis by surface plasmon resonance also showed that the kinetics of the antigen-binding were essentially unchanged. Although both the scFv and ds-Fv existed predominantly in the monomeric form, trace amounts of dimer were removed by HPLC prior to surface plasmon resonance analysis. Dimerization is frequently seen with scFv molecules [16] and has an especially significant effect on the binding properties of this antibody because of the multivalent nature of its carbohydrate antigen. Both the association and dissociation phases were fitted well by the BIAevaluation 2.0 one-component interaction model. The association rate constants were determined to be $6.5 \times 10^4$ and $7.2 \times 10^4$ $M^{-1}s^{-1}$ for scFv and ds-scFv, respectively. Both forms displayed rapid and very similar dissociation rate constants, 0.11 and 0.15 $s^{-1}$. The $K_a$ values of $5.9 \times 10^5$ and $4.8 \times 10^5$ $M^{-1}$ are in good agreement with the value for the scFv from titration microcalorimetry [9].

### 3.3. Thermal stability of the ds-scFv

The thermal denaturation of the disulphide-bonded molecule was compared with that of the parent scFv and Fab by monitoring the amide I′ bands in their FTIR spectra, at various temperatures. This band primarily reflects the peptide C=O stretching vibration which is very sensitive to hydrogen bonding and thus protein secondary structure. At temperatures below the thermal transition, the spectra of all three samples exhibited an asymmetric band contour with intense maxima near 1635 $cm^{-1}$ and a weak shoulder near 1680 $cm^{-1}$ (Fig. 4), indicative of predominantly $\beta$-sheet proteins [17,18]. Upon heating all three samples, a gradual increase in the intensity of the 1635 $cm^{-1}$ vibration was observed, concomitant with increases at 1690 and 1620 $cm^{-1}$. The latter changes have been noted previously in thermal denaturation spectra and are attributed to protein aggregation [19,20]. The thermal denaturation curves obtained from the changes in intensity at 1620 $cm^{-1}$ are shown in Fig. 5. It is immediately evident that the ds-scFv has considerably greater thermal stability than the parent scFv, the two $T_m$ values being approximately 60°C and 69°C. The Fab fragment of Se155–4 had a $T_m$ of 75°C.

## 4. Discussion

The weak interaction between $V_L$ and $V_H$ domains is a major concern in therapeutic applications of Fvs and Fv immunotoxins. There appears to be a wide range of interaction strengths and in one case there was little association at all [21], while in another the addition of isopropanol disrupted it extensively [22]. Hence, stabilization by scFv or dsFv approaches has been extensively pursued. In the experiments presented here, we combined these approaches and created a ds-scFv species. This construct is superior to the two individual approaches in that it combines the merits of both. The linker peptide of the scFv element dictates coordinated expression of the two domains, and reduces the possibility of homodimer formation during refolding and oxidation to form the disulphide bridge. The disulphide feature brings enhanced thermal stability and also overcomes two disadvantages of scFvs. These are their tendency to dimerize, which greatly affects biophysical measurements of their binding properties (C.R. MacKenzie et al., submitted for publication) and the equilibrium they presumably have between 'open' and 'closed' forms, i.e. with and without $V_L$-$V_H$ pairing. The latter phenomenon is probably responsible for some Fvs showing lower affinities for antigen than the corresponding Fabs.

The sites chosen for the two cysteines in the present construct are close to those in one of the two designs of Pastan and coworkers, L43-H105 (Kabat numbering) [3–5]. The construct differs in that the L chain Cys at residue 57 (L55 in Kabat numbering) is at the end of, rather than before, the CDR L2, and the H chain Cys at position 106 (108 in Kabat numbering) is positioned four residues earlier in the sequence. The H-chain Cys is at a position where an Asp required for a salt-bridge to Arg-H98 (94 in Kabat numbering) is often found, so this was compensated for by altering the next residue to Asp, a mutation previously shown to retain full antigen-binding activity in Fab [15]. The position of the disulphide bond is closer to the binding-site than in the Jung et al. design [3] but not as close as in the design of Glockshuber et al. [2], which involves CDR H3 residues. The $C_\alpha$-$C_\alpha$ distance between our introduced Cys residues varies between 5.76 and 6.12 Å among the five different

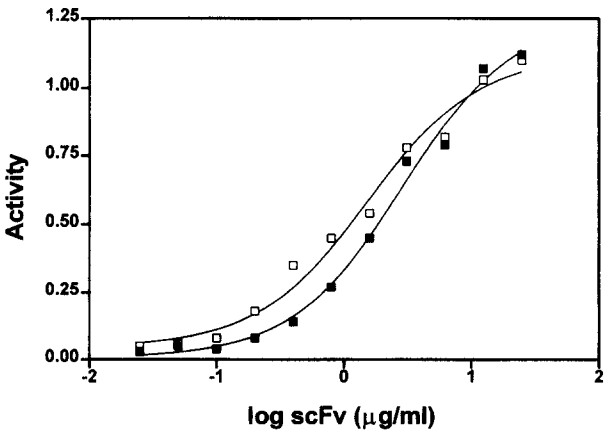


Fig. 3. Indirect EIA showing the binding of Se155–4 scFv and ds-scFv to BSA-*O*-polysaccharide. Open squares, scFv; solid squares, ds-scFv.

crystal structures solved for Se155–4. The largest distance was observed in the scFv and is slightly longer than the longest distance spanned by one of the introduced disulphide bonds by Jung et al. [3]. Our analysis of possible sites for introduction of the disulphide bond did not detect their L43-H105 site (Kabat numbering), as this distance is 7.26 Å in Se155–4, due in part to differences in the conformation of the light chain $\beta$-strand at this point. Their second design, L106-H44, has a corresponding distance of 6.19 Å the scFv crystal structure and, therefore, may also work in Se155–4.

However, our design could not be used in a structure based on M603 (the template used by Jung et al. [3]), as the $C_\alpha$-$C_\alpha$ distance between corresponding residues is 9.12 Å, rather than the 6.12 Å measured in the scFv of Se155–4. Two structural factors appear to contribute to this longer distance, viz. the conformation of the L2 loop in relation to the rest of the VL domain and its possible dependence on H3 loop conformation. Although the inner $\beta$-strands in the VL domain which form the VL:VH interface are relatively invariant in their $\beta$-strand conformation, the $\beta$-strand leading into the L2 loop of M603 bends back and away from the VL:VH interface much more than the corresponding strand in Se155–4. This conformation may be a structural adaptation to the conformation of the H3 loop in M603, to which it is adjacent. In Se155–4, the reverse situation holds, with the L2 loop folding closer to the VL:VH interface and the H3 loop folding out of the way, in this case, towards the central pocket of the combining site. It should also be noted that Se155–4 has a $\lambda$ light chain rather than the more common $\kappa$ light chain, but it is not possible to say whether other, more subtle, structural differences between the two light chain types are influencing the distance between VL and VH residues.

The immunological assays, EIA and surface plasmon resonance, showed the ds-scFv also was fully active. However, the mutations had a deleterious effect on functional expression in the periplasm, as is often the case when Cys residues are introduced into this scFv. Since many scFv and other dsFv species are prepared from inclusion bodies, this is not a particular problem.

Introduction of disulphides into proteins has frequently been used to enhance their stability. Assessment of the change in stability of Fv produced by this means has usually been done by non-biophysical methods, such as retention of immunological activity after heating [2,5]. Such experiments showed that disulphide bonds enhanced stability as expected, but $T_m$ values were not obtained. The FTIR approach used here has the advantage of providing a good biophysical measurement of thermal stabilization, while requiring very little material. The scFv denatured at 60°C, a temperature close to the 62°C found for an antilysozyme Fv [23] using circular dichroism, but above the value for an antidansyl Fv of 52.3°C [24] obtained by differential scanning calorimetry. While the $T_m$ of the ds-scFv was increased by 9°C, the Fab still had a higher value, 75°C. The 15°C difference between the Fv $T_m$ and Fab $T_m$ is the same as in the antidansyl antibody [24]. Yasui et al. [23] also observed $V_L$-$V_H$ dissociation at lower temperatures and found that single mutations in the CDRs could dramatically lower the thermal stability of their Fv. Since the interaction between $V_L$ and $V_H$ involves residues from the CDR loops, each Fv can be expected to have a unique $T_m$ and $V_L$-$V_H$ dissociation behaviour. Nevertheless, the increase in $T_m$ of 9°C for the ds-scFv shows that introduction of a disulphide into an scFv should be a valuable general route to Fv molecules of enhanced stability.

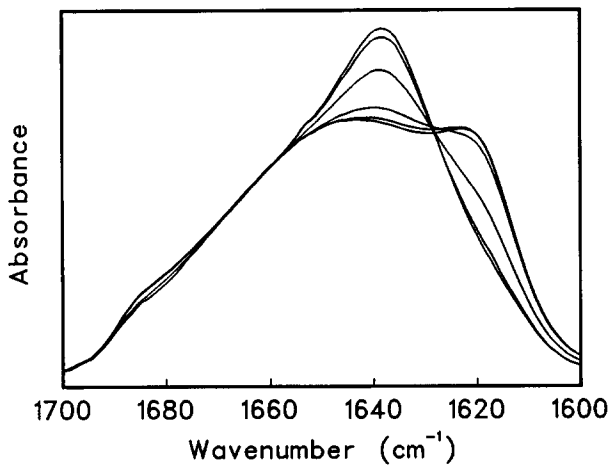


Fig. 4. FTIR spectra of Se155–4 ds-scFv in $^2H_2O$ as a function of temperature. The spectra were recorded at 69, 72, 75, 77, 80 and 83°C (top to bottom at the 1635 cm$^{-1}$ peak). The $^2H_2O$ vibrations have been subtracted and the spectral baselines were corrected between 1730 and 1590 cm$^{-1}$.

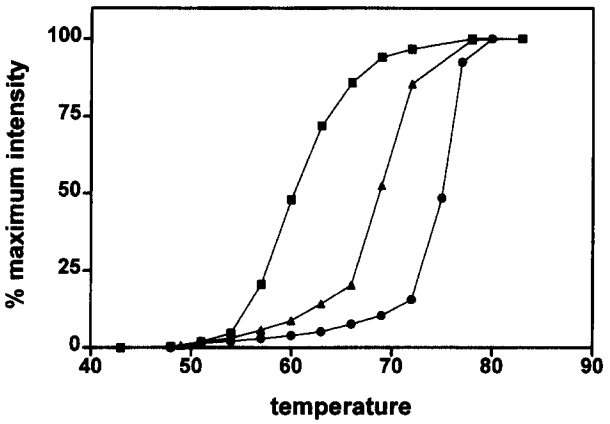


Fig. 5. Thermal denaturation curves calculated from the FTIR spectra for Se155–4 scFv (■), ds-scFv (▲) and Fab (●).

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