Expression, purification and characterization of B72.3 Fv fragments

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The Fv fragment of the antibody B72.3 has been produced by expression in both a mammalian and microbial system, namely Chinese hamster ovary (CHO) cells and *Escherichia coli*. In both cases secretion of the Fv into the culture medium was achieved, with equivalent amounts of Vh and Vl produced. The yield of Fv from CHO cells was 4 mg/l in roller-bottle culture. *E. coli* proved to be a more productive system with yields of 40 mg/l in shake flasks rising to 450 mg/l in fermentations. B72.3 Fv from both sources was capable of binding to antigen with similar binding ability to the Fab′ fragment. A detailed sedimentation analysis, both by velocity and equilibrium techniques, revealed that the two domains of Fv are associated at high concentrations at pH values close to neutral, but dissociate at concentrations lower than approx. 0.5 mg/ml. Individual Vh or Vl polypeptides are not able to bind to the antigen and thus these results suggest that the antigen promotes assembly of Fv at the low concentrations used in the antigen-binding assays. At a pH value of 1.9, Vh and Vl are completely dissociated even at very high concentrations and are apparently unfolded at low solute concentrations. Small-angle X-ray scattering was used to measure a radius of gyration of 1.75 ± 0.2 nm (17.5 ± 2 Å) for Fv.

INTRODUCTION

IgG molecules are made up of four polypeptide chains, two heavy chains of approx. 55000-M_r and two light chains of approx. 28000-M_r. These polypeptide chains are folded into domains with a common fold comprising two β-sheets stabilized by an intra-domain disulphide bond [1]. Comparison of sequences of other proteins has now revealed that the 'immunoglobulin fold' is present in a large number of other proteins which have been grouped together to form the Ig superfamily [2]. Many of the members of the Ig superfamily are of biological importance and thus there is great interest in the study of their structure and function. Structural studies of these molecules are often difficult to carry out because of their large size, complexity and the presence of flexible sequences joining one domain to another. However, individual Ig domains are amenable to structural analysis [3–5]. This requires the production of large amounts of protein, which in turn requires an efficient expression system.

The antigen-binding region of an Ig molecule resides entirely in the paired variable domains of the heavy- and light-chains (Vh and Vl) which together make up an Fv. The preparation of Fv fragments was first described by digestion of a murine myeloma IgA, MOPC315, with pepsin [6,7]. However, attempts to prepare Fv fragments of other antibodies by proteolysis have proven largely unsuccessful, being limited to a small number of cases of digestion of IgA myeloma protein [8], IgG containing λ light-chain [9], and IgG2a with a deleted CHl domain [10]. With the advent of recombinant DNA technology the direct expression of Vh and Vl has been possible. Intracellular expression of Fv in *Escherichia coli* has been demonstrated for an anti-lysozyme antibody, gloop 2 [11], as has intracellular expression of individual Vh and Vl domains [12]. However, the advantages of high expression levels were offset by the need to solubilize and refold the Fv polypeptides to obtain antigen-binding activity. As an alternative, expression of Fv of the IgA myeloma antibody McPc603 has been achieved using secretion from *E. coli* as the expression system [13]. In this case low yields were reported of 0.2 mg/l which could be raised only to approx. 0.5 mg/l by the design of improved vector systems [14]. Only one example of Fv expression in mammalian cells has been reported. The Fv of the anti-lysozyme antibody D1.3 has been expressed in myeloma cells as a humanized version with a yield of 8 mg/l [15] and also in *E. coli* as the murine Fv, with a yield of 10 mg/l [16].

The variable, and often low, yields observed for expression of Ig domains make it difficult to decide upon the best expression system to use to produce Fv fragments. Variation is seen between different expression systems and between Fv fragments of different antibodies. For this reason we have compared directly the expression of the same Fv fragment in Chinese hamster ovary (CHO) cells and in an *E. coli* secretion system. We chose the Fv of the antibody B72.3 directed towards a tumour-associated antigen [17]. The cloning of the heavy- and light-chain (*Vh* and *Vl*) genes for B72.3 has been described [18] as has the expression of a mouse–human chimeric version of the B72.3 antibody and a chimeric Fab′ fragment in CHO cells [19,20]. The development of a high-yielding expression system has now allowed us to characterize the interaction between the domains of the Fv in more detail than has been possible previously.

MATERIALS AND METHODS

Vector construction

The cDNA of the light- and heavy-chains of B72.3 [18], were subjected to site-directed mutagenesis [21] to introduce *Eco*R1 restriction sites and translation stops at the 3′-ends of the variable domains. In addition, to facilitate the manipulation of

Abbreviations used: Vh, variable domain of the IgG heavy-chain; Vl, variable domain of the IgG light-chain; Fv, paired variable domains of the IgG heavy- and light-chains; CHO, Chinese hamster ovary; MSX, methionine sulphoximine; IPTG, isopropylthiogalactoside; s.a.x.s., small-angle X-ray scattering; scFv, single-chain Fv; OmpA, outer-membrane porin A; HRP, horseradish peroxidase.

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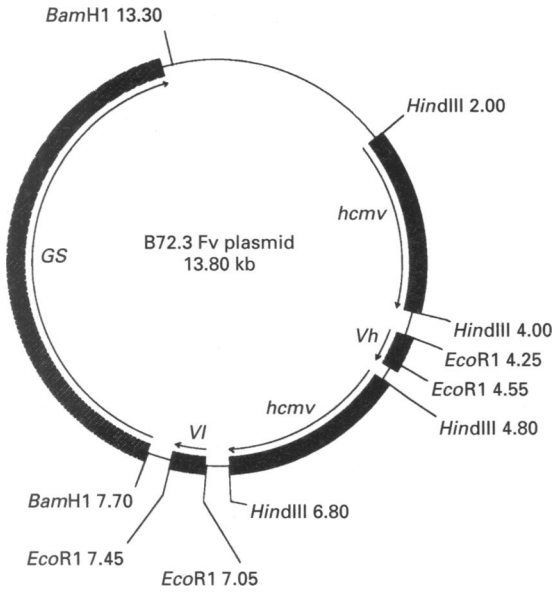

Figure 1 Map of B72.3 Fv CHO expression vector

Abbreviations used: *hcmv*, human cytomegalovirus promoter; *GS*, glutamine synthetase selectable/amplifiable marker, *Vl*, light-chain variable region gene; *Vh*, heavy-chain variable region gene. The positions of relevant restriction enzyme sites are indicated.

the variable gene sequences, *Eco*RV and *Hin*dIII sites were also incorporated into *Vl* to match the naturally occurring *Pvu*II and *Bg*l1 sites in *Vh*. The genes were cloned either separately or in tandem, into an expression vector under the control of the human cytomegalovirus promoter with a simian virus-40 polyA addition sequence [22]. A construct, with the genes arranged in tandem, is shown in Figure 1. This plasmid also contains the glutamine synthetase gene as a selectable marker [23]. The B72.3 Fv constructs were tested by transient expression in COS1 cells [18]. Synthesis and secretion of variable domains was assayed by biosynthetically labelling the transfected cells with [^{35}S]-methionine (100 μCi/10^6 cells per ml for 48 h). The cell supernatants were subjected to immunoprecipitation with antisera reacting with either Vl- or Vh-reactive epitopes. Vh and Vl domains were detected in COS-cell supernatants as polypeptides of M_r 14000 and 12000 after both reducing and non-reducing SDS/PAGE (compared with values calculated from the deduced amino-acid sequence of 12600-M_r and 11800-M_r for Vh and Vl respectively). Apparently, higher levels of expression were obtained from the dual *Vh/Vl* plasmid compared with single *Vh* or *Vl* plasmids. These results were taken to indicate that the assembled constructs were capable of expressing the correct Vh and Vl polypeptides.

Expression in CHO cells

CHO K1 cells were grown in Glasgow-modified Eagle's medium (Gibco), supplemented with non-essential amino acids (Gibco), 30 μM adenosine, 30 μM guanosine, 30 μM cytidine, 30 μM uridine, 10 μM thymidine, 500 μM glutamate, 500 μM asparagine (Sigma), 1 mM sodium pyruvate, 50 units/ml penicillin/streptomycin and 10% (v/v) dialysed foetal calf serum (Gibco). The *Vh/Vl* construct described for transient expression was transfected into CHO K1 cells by calcium phosphate precipitation as described by Bebbington [24]. Plasmid DNA (10 μg) as a calcium phosphate–DNA precipitate, was added to 10^6 cells/90 mm diam. Petri dish. After 24 h transfected cells were selected by adding methionine sulphoximine (MSX) to the medium (25 μM final concentration). Twenty-four resistant colonies observed after 15 days were picked, expanded and the cell media assayed for B72.3 Fv production by Western blotting using rabbit anti(B72.3 Fab′) serum [20]. Fv-producing cell lines were amplified by selection with increasing amounts of MSX [23]. Colonies obtained at the highest MSX concentration (500 μM) were screened for Fv production by Western blotting. The two cell lines secreting the most Fv as judged by Western blotting were grown to 1 l cultures in roller bottles and productivity assessed after purification of the secreted Fv.

Expression of *E. coli*

Secretion of Vl and Vh from *E. coli* was directed by the outer-membrane porin A (*OmpA*) signal sequence [25]. A 92 bp fragment comprising the natural translation initiation and signal sequence encoding sequences of *OmpA* was assembled from oligonucleotides and cloned between the *Xho*1 and *Hin*dIII sites of pSK$^+$ (Stratagene Cloning Systems) to give the plasmid pSKomp. The *Hin*dIII site in this plasmid is located at the 3′-end of the signal sequence. pSKomp was cleaved with *Hin*dIII and *Eco*R1 and the *Eco*RV–EcoR1 fragment encoding most of *Vl* cloned into it using oligonucleotide adaptors to give a precise *OmpA–Vl* fusion. Similarly a precise OmpA–Vh fusion was constructed using adaptors between the *Hin*dIII site of the signal sequence and the *Pvu*II site in *Vh*. The *OmpA–Vl* fusion was cloned as a *Xho*1–*Eco*R1 fragment into plasmid pTTQ9 (Amersham International) cleaved with *Sal*1 and *Eco*R1 to give plasmid pTTQ9–Vl. The *OmpA–Vh* fusion was excised as a *Xho*1–*Eco*R1 fragment, the ends filled in using the Klenow fragment of DNA polymerase and the fragment cloned into pTTQ9-Vl cleaved with *Eco*R1 and with the end filled in. A plasmid with the *OmpA–Vh* fusion oriented to give appropriate transcription from the tac promoter along with *OmpA–Vl* was designated pTTQ9-Vl/Vh. A fragment carrying a *Kan^r* gene was cloned from plasmid pUC-4K (Pharmacia) into the *Sca*1 site in the *Amp^r* gene of pTTQ9-Vl/Vh to serve as an efficient selectable marker. This final plasmid for expression and secretion of the B72.3 Fv in *E. coli* was designated pCT001.

E. coli strain XL1Blue was used as the host strain for both construction of pCT001 and the expression of the Fv. For expression in shake flask XL1Blue (pCT001) was grown in 50 ml of L-broth (comprising, per litre, 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl) containing 30 μg/ml kanamycin in a 250 ml baffled flask at 30 °C with shaking. At a culture A_{600} of 0.5, expression of the *Fv* was induced by adding isopropyl-thiogalactoside (IPTG) to a final concentration of 1 mM. Fv was recovered from the culture supernatant after a further 12–18 h of incubation. For larger scale production XL1Blue (pCT001) was grown at 30 °C in a 2 l MBR minibioreactor in YEGLY medium containing kanamycin at 30 μg/ml. YEGLY medium comprised per litre: 7 g of ammonium sulphate, 6.24 g of sodium dihydrogen phosphate, 40 g of yeast extract, 20 g of glycerol and 10 ml of a trace-element solution comprising (per litre), EDTA, 60 g, $MgSO_4,7H_2O$, 20 g; $CaCl_2,6H_2O$, 5 g; $ZnSO_4,4H_2O$, 2 g; $MnSO_4,4H_2O$, 2 g; $CuSO_4,5H_2O$, 0.5 g; $CoCl_2,6H_2O$, 0.095 g; $FeSO_4,7H_2O$, 10 g and NaOH, 15 g. The pH value in the fermenter was maintained at pH 7 by addition of 2 M NaOH. Expression of the Fv was induced in the fermentor at an A_{600} of 10 and the Fv recovered from the culture supernatant after a further 12–18 h of incubation.

Purification of B72.3 Fv

Fv fragments expressed by CHO cells or *E. coli* were purified by affinity chromatography on mucin–Sepharose. Bovine submaxillary mucin (Boehringer) was coupled to CNBr-activated Sepharose (Pharmacia) by standard techniques at 30 mg per ml of Sepharose. Cell supernatants (CHO or *E. coli*) were clarified by centrifugation and concentrated by ultrafiltration to one-tenth of the original volume using an Amicon stirred-cell system with a YM5 membrane. Concentrated supernatants were applied to a column of mucin–Sepharose pre-equilibrated with PBS, pH 7.4. Following extensive washing with at least 10 column volumes of PBS, bound material was eluted from the column with 0.1 M citric acid. Fractions from the column were collected directly into sufficient 1 M Tris to adjust the pH to a value of 7–7.5. Fractions containing Fv were pooled, concentrated to approx. 1 mg/ml and dialysed into 50 mM phosphate buffer, pH 7.4.

SDS/PAGE was carried out according to Laemmli [26] using 15% (w/v) acrylamide gels.

Antigen-binding analysis

For recognition of Fv samples antiserum was produced from rabbits using murine Fab as immunogen. Any recognition of human constant regions was removed by pre-absorption of the antiserum by passing through a column of human IgG4–Sepharose (produced by coupling human IgG4 to CNBr-activated Sepharose). This antiserum was then used to compare the antigen-binding properties of Fv to chimeric Fab fragments using a direct-antigen-binding e.l.i.s.a. carried out as described previously [20]. Competition e.l.i.s.a. was also carried out as described previously [27]. Briefly, the Fv and Fab samples were competed with horseradish peroxidase (HRP)-labelled B72.3 IgG for binding to solid-phase mucin. Signal was detected by measuring the amount of HRP-labelled B72.3 bound with tetramethylbenzidine substrate.

Preparation of samples for sedimentation studies

Sedimentation studies were carried out at two pH values, 6.8 and 1.9. Initially fragments were dialysed extensively [28] against buffers at the appropriate pH value either phosphate/chloride, pH 6.8, 0.1 M (4.595 g of Na_2HPO_4,12H_2O, 1.561 g of KH_2PO_4 and 2.923 g of NaCl per litre [29]) or KCl/HCl, pH 1.9, 0.2 M (14.919 g of KCl and 65 ml of 0.2 M HCl per litre). Samples were centrifuged in a Beckman preparative ultracentrifuge with a 70Ti rotor at 126000 *g* for 20 min to remove any dust or fine precipitate, although any large contaminants are rapidly sedimented to the cell base during an analytical sedimentation experiment.

Sedimentation velocity

Sedimentation velocity experiments were performed using an MSE Centriscan 75 analytical ultracentrifuge equipped with scanning absorption and schlieren optics and a monochromator. The rotor speed was between 40000 and 50000 rev./min depending on the rate of solute sedimentation. Traces were analysed on a digitizing pad connected to a computer equipped with software to generate sedimentation coefficients at concentrations corrected for radial dilution. These coefficients were then corrected to standard conditions (water at 20 °C) [30] and plotted as a function of solute concentration. A partial specific volume of 0.727 ml/g for Fv was estimated from the amino-acid sequence using Traube's rule [31].

Sedimentation equilibrium

Sedimentation equilibrium was performed using an MSE Centriscan 75 analytical ultracentrifuge [28] in order to establish the dependence of relative molecular mass on solute concentration and solution pH. This was used in preference to the Beckman model E analytical ultracentrifuge because the sedimentation process can be continually monitored without the need to take a series of photographs; four samples can be analysed at a time without the need for multichannel cells or wedge windows and samples of lower concentration can be used.

Scanning-absorption optics were used to follow the course of the sedimentation and eventual equilibrium, which was generally achieved within 24 h. The rotor speed was 25000 rev./min and the runs were conducted at 20.0 °C for solutions at pH 6.8 and 6.0 °C for more acidic pH values when the fragments tended to thermally degrade. Cells and solutions were prepared as for sedimentation-velocity experiments. Traces were digitized and then analysed using simple linear regression analysis software written by Dr. A. Rowe, Department of Biochemistry, University of Leicester, Leicester, U.K.

The whole-cell weight average relative molecular mass ($\bar{M}^0_w$) was obtained at several solute concentrations at both pH 6.8 and pH 1.9 from the extrapolation of the star-average relative molecular mass ($\bar{M}^*$) versus ξ plot obtained from sedimentation equilibrium experiments following the method of Creeth and Harding [32]:

$$\bar{M}^*(\xi \rightarrow 1) = \bar{M}^0_w$$

where $\xi = (r^2 - a^2)/(b^2 - a^2)$, r = radial displacement from the centre of the rotor, a = radial position of meniscus and b = radial position at the base of the cell.

Small-angle X-ray scattering (s.a.x.s.)

Preliminary s.a.x.s. measurements at pH 6.8 were performed on Station 2.1 at the Synchrotron Radiation Source, Daresbury Laboratory, Daresbury, Cheshire, U.K. A camera length of approx. 5 m was employed and the sample was housed in a motor-driven translating cell in order to provide a statistically significant number of counts at each concentration. The system was calibrated by acquiring the well-characterized profile of dry collagen and results were corrected for background-scattering and non-protein-scattering contributions by collecting the spectrum of the dialysate alone.

Data analysis was performed at Daresbury using in-house software. The radius of gyration was obtained using the Guinier relation (e.g. [33])

$$\ln I(s) = \ln I(0) - 4/3\pi^2 r_g^2 s^2$$

where I is the measured photon intensity at scattering angle 2θ, $s = 2 \sin \theta/\lambda$, λ is the wavelength of synchrotron radiation and r_g is the radius of gyration. r_g was measured at three concentrations and extrapolation to infinite dilution yielded a measure of $r_g(0)$ for Fv.

RESULTS

Expression and purification of B72.3 Fv

Expression of B72.3 Fv was achieved with both CHO cells and *E. coli*, the Fv being secreted into the culure medium of both cell types. Secretion of Vh and Vl occurred in approx. equal amounts with no excess of any one chain observed. Purification was achieved by affinity chromatography using bovine submaxillary mucin coupled to CNBr-activated Sepharose. Bovine sub-

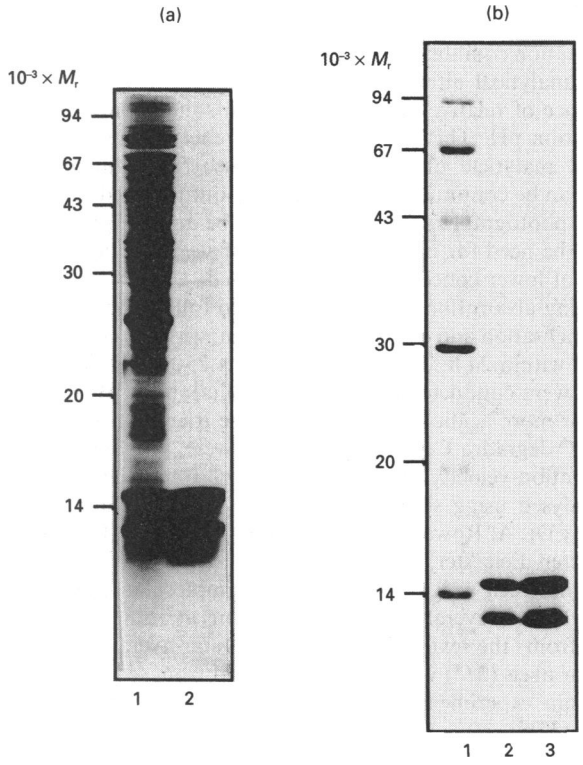

Figure 2 SDS/PAGE of B72.3 Fv

SDS/PAGE was carried out on 15% (w/v) gels under reducing conditions. (**a**) Purification of B72.3 Fv from *E. coli* culture supernatant. Lane 1, *E. coli* culture supernatant loaded on to a mucin–Sepharose column; lane 2, mucin–Sepharose column eluent. Positions of marker proteins are indicated. (**b**), B72.3 Fv purified from CHO cells and *E. coli*. Lane 1, marker proteins (M_r indicated); lane 2, CHO-derived Fv; lane 3, *E. coli*-derived Fv.

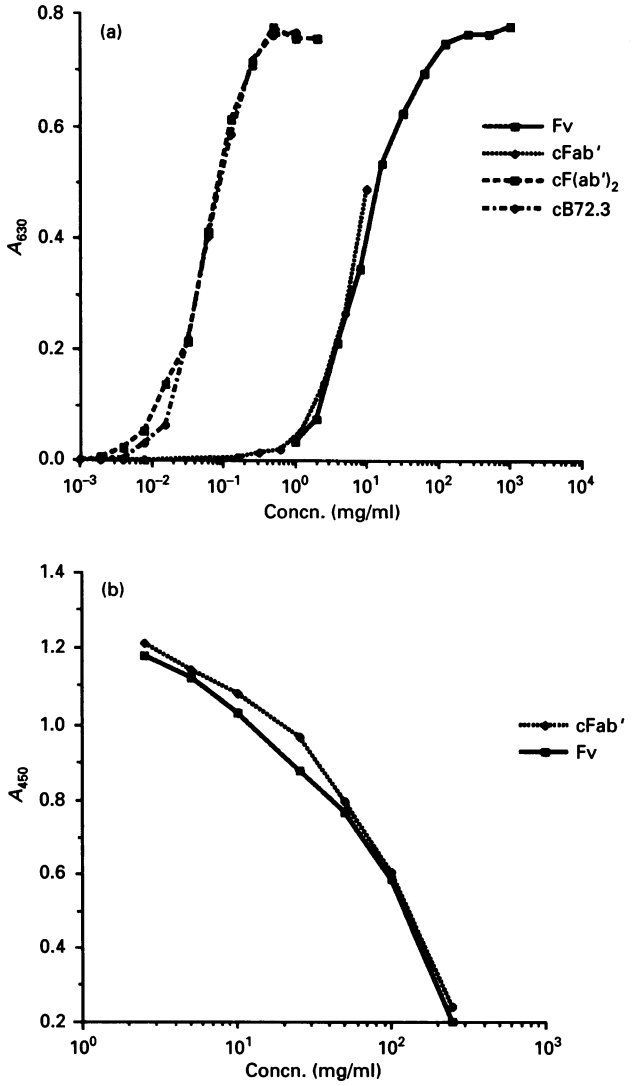

Figure 3 Relative antigen binding of B72.3 Fv

(**a**) Direct binding and (**b**) competitive e.l.i.s.a. of B72.3 Fv compared to cFab' fragment and divalent species [cB72.3 IgG and cF(ab')$_2$]. The concentrations of Fab' and Fv were determined by measuring absorbance of 280 nm using a specific absorbance value $A^{1\%}_{1\,cm}$ of 11.4.

maxillary mucin has been shown to contain a glycopeptide which is recognized by B72.3 due to its similarity to the epitope recognized by B72.3 on the tumour-associated glycoprotein TAG72 [34]. Purification of B72.3 Fv was efficient from both CHO cell supernatant and *E. coli* supernatant, with > 90% purity achieved in a single step (Figure 2). No Vh or Vl could be detected in the flow through from the column by SDS/PAGE or Western blotting, suggesting that all of the Vh and Vl present was capable of recognizing the mucin and would therefore be expected to recognize the B72.3 antigen.

The yield of Fv from both types of expression system was measured as the amount of Fv which could be purified in each case. CHO cells secreted only very small amounts of Fv before amplification, but amplified CHO cell lines were identified which secreted 4 mg/l of supernatant in roller-bottle culture. With *E. coli* yields of 40 mg/l of supernatant were seen in shake-flask cultures, and in fermentation cultures yields of 450 mg/l were seen. These yields compare favourably with those seen by other workers [11,13–16].

Characterization of B72.3 Fv

SDS/PAGE analysis of the purified Fv from CHO cells and *E. coli* revealed the expected polypeptides at approx. M_rs of 12000 and 14000 corresponding to Vl and Vh (Figure 2b). The identities of these bands were confirmed by N-terminal-sequence analysis directly from a blotted SDS/acrylamide gel. The sequence of the first 15 residues was obtained in each case. This revealed an identical sequence to that predicted from the DNA sequence for both Vh and Vl, indicating correct and complete removal of the signal sequence.

The antigen-binding activity of the purified Fv was measured using both a direct antigen-binding e.l.i.s.a. and a competitive e.l.i.s.a. (Figure 3 and [35]). Controls demonstrated that there was no binding to either the plate itself or casein used as a blocking agent in these assays. Results from the direct binding e.l.i.s.a. revealed apparently similar binding ability of the purified Fv with the chimeric Fab' fragment (Figure 3a). The chimeric Fab' fragment has identical antigen-binding properties to the murine Fab [20]. Bound Fv and chimeric Fab' were detected using polyclonal antiserum raised against murine Fab'. This reagent detects the murine variable regions which are in common between these molecules. A similar result was also seen with the competition assay, carried out as described previously [35] and Figure 3(b), with equivalent competition of the Fv and Fab' fragment. Results are plotted in mg/ml protein, assuming an

identical specific absorbance for the Fab′ and Fv of $A^1_{1cm} = 11.4$. This specific absorbance has been measured for the Fab′ using quantitative amino-acid analysis (D. J. King and A. Carne, unpublished work).

The individual domains of the Fv, Vh and Vl were also produced, both by separate expression in *E. coli* and by separation of the purified Fv either by ion-exchange chromatography or by reverse-phase h.p.l.c. In neither case could any antigen-binding ability of the isolated domains be detected either in direct binding or competition assays (results not shown). These results therefore suggest that in the conditions of these assays the Fv must be assembled into the Vh–Vl heterodimer.

Sedimentation analysis

Sedimentation-velocity experiments were carried out over a range of concentrations at values of both pH 6.8 and 1.9. Standardized $s_{20,w}$ values were calculated and are plotted against concentrations corrected for radial dilution in Figure 4 for pH 1.9 and pH 6.8. The similarity of these curves to those described by Gilbert and Gilbert [36], suggests that a transient, reversible dissociation of the Fv domains is taking place.

Extrapolation to infinite dilution of the results in Figure 5 provides an estimate of the weight average relative molecular mass of the solute species in almost ideal conditions ($\bar{M}_{r,w}$). At neutral pH values $\bar{M}_{r,w} = (22300 \pm 1500)$ g/mol which compares favourably with a M_r value of 24451 g/mol calculated from the amino-acid sequence of Fv (M_r 12604 g/mol for Vh, M_r 11847 g/mol for Vl). At a pH value of 1.9 $\bar{M}_{r,w} = (14300 \pm 1500)$ g/mol, suggestive of a predominantly dissociated solute species.

It is possible to use the Svedberg equation to gain insight into the extremes of Fv conformation under the pH and concentration regimes imposed.

The frictional ratio for a solute species given in eqn. (1):

$$\frac{f}{f_0} = \frac{M(1-\bar{\nu}\rho)}{N_A s^0 6\pi\eta\left(\frac{3M\bar{\nu}}{4\pi N_A}\right)^{\frac{1}{3}}} \quad (1)$$

where f is the frictional coefficient of the molecule, f_0 is the frictional coefficient of a sphere with volume equal to that of the anhydrous molecule which has a partial specific volume of $\bar{\nu}$, a molecular mass of M and a sedimentation coefficient of s^0. The density and viscosity of the solvent is given by ρ and η respectively. N_A is Avogadro's number and can be broken down into a contribution from shape (P, the Perrin function) and the effect of hydration (f/f_h) (see, for example, [37]).

$$\left(\frac{f}{f_0}\right) = P\left(\frac{f}{f_h}\right) = P\left(\frac{\nu_s}{\bar{\nu}}\right)^{\frac{1}{3}} \quad (2)$$

In eqn. (2) f_h is the frictional coefficient of an anhydrous molecule of a shape identical to that of the molecule in question which has a solvated specific volume of $\nu_s = \bar{\nu} + \delta\nu^0$, δ being the degree of molecular hydration (in g of water/g of protein) and ν^0 the partial specific volume of water.

The maximum hydration of the molecule can then be estimated by minimizing the contribution to the deviation from spherity (i.e. by setting $P = 1$ which corresponds to a sphere)

$$\delta_{max.} = \frac{1}{\nu^0}\left\{\frac{4\pi N_A}{3M}\left[\frac{M(1-\bar{\nu}\rho)}{6\pi\eta N_A s^0}\right]^3 - \bar{\nu}\right\} \quad (3)$$

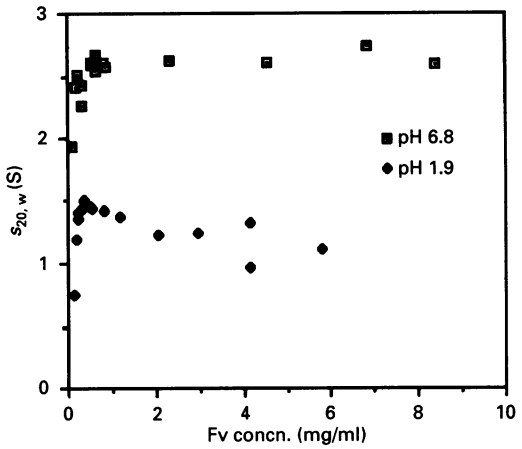

Figure 4 Effect of concentration on the sedimentation coefficient of Fv

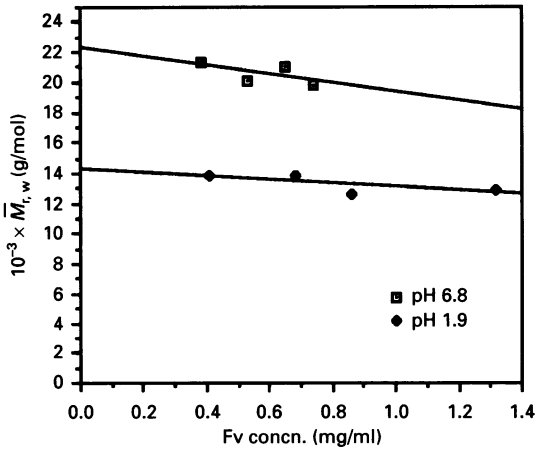

Figure 5 Effect of concentration of $M_{r,w}$

Table 1 Interpretation of hydrodynamic data in terms of maximum molecular hydration

M_r values were determined from amino-acid sequences.

pH value	Concentration range (mg/ml)	M_r (g/mol)	$s^0_{20,w}$(s)	$\delta_{max.}$* (g of solvent/g of protein)
6.8	⩾ 0.5	24451	2.60 ± 0.2	0.37 ± 0.25
	< 0.5	24451	2.00 ± 0.3†	1.69 ± 1.09
			1.40 ± 0.3‡	6.33 ± 2.96
		12226	2.00 ± 0.3†	−0.12 ± 0.27
			1.40 ± 0.3‡	1.04 ± 1.14
1.9	⩾ 0.5	24341	1.46 ± 0.2	5.94 ± 1.77
		12226	1.46 ± 0.2	0.94 ± 0.69
	< 0.5	12226	0.7 ± 0.3	14.39 ± 19.44

* As defined in eqn. (3).
† Linear extrapolation of data.
‡ Non-linear extrapolation of data.

These parameters, as calculated from experimental data, are shown in Table 1. For concentrations below 0.5 mg/ml at pH 6.8 molecular masses calculated from the amino-acid sequence for

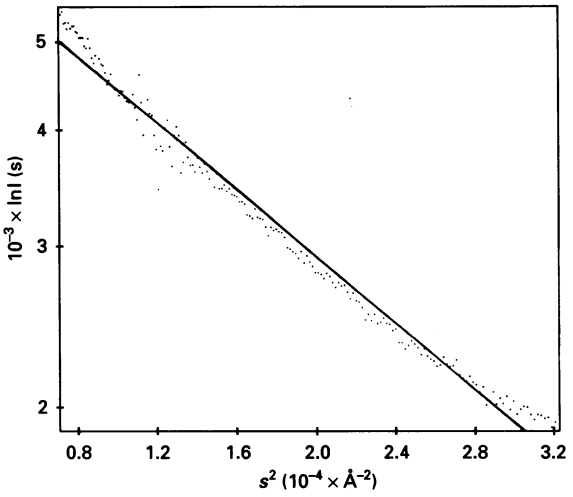

Figure 6 Guinier plot for Fv at a concentration of 10 mg/ml

Guinier plot of ln (intensity) versus angle of scatter in s-space. The solid line corresponds to r_g of 1.79±0.02 nm (17.9±0.2 Å). s is related to the conventional scattering angle, 2θ, by $s = (2 \sin\theta)/\lambda$, where λ is the wavelength of synchrotron radiation.

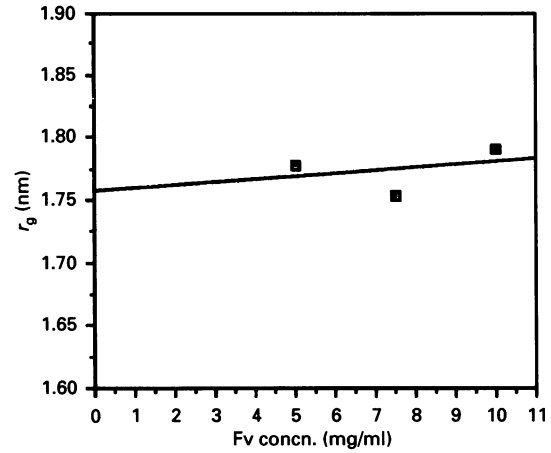

Figure 7 Effect of concentration on r_g

Plot of apparent r_g versus concentration for Fv at pH 6.8. Results were collected for at least 20 min per point with count rates of between 3.9 kHz and 5 kHz and a synchrotron current of 110–112 mA.

both Fv and its domains have been used in calculations for comparative purposes. The need for this at a value of pH 1.9 is ruled out by the sedimentation equilibrium data which clearly point to a monomer molecular mass. Thus it can be surmised that at pH 6.8 Fv exists as the heterodimer of Vh and Vl at concentrations above 0.2–0.5 mg/ml. Below this it is most likely that Fv reversibly dissociates into individual domains with a small change in $\delta_{max.}$.

At a value of pH 1.9, the dissociated Fv is moderately hydrated, but this changes drastically at low concentrations when the maximum hydration could be as high as 14.4 g of water/g of protein (see Table 1). It is likely therefore that the protein has unfolded, or that the observed reduction in sedimentation coefficient is due to an increased asymmetry of the solute species. Glockshuber et al. [38] have reported that the Fv of the murine IgA McPC603 is unstable at low protein concentrations.

S.a.x.s.

In order to fully interpret sedimentation velocity results in terms of conformation, a measure of molecular dimension such as r_g is required. The small size of Fv and its constituent polypeptides precludes the measurement of r_g by light scattering but preliminary s.a.x.s. measurements were made at pH 6.8.

Figure 6 shows a Guinier plot obtained at an Fv concentration of 10 mg/ml. Results were collected for 20 min. Similar plots were obtained at concentrations of 5.0 and 7.5 mg/ml and their extrapolation to infinite dilution is shown in Figure 7. This yields a value for $r_g(0)$ of 1.75±0.2 nm (17.5±2 Å). Measurements at pH 1.9 have yet to be performed.

DISCUSSION

By comparing the expression and secretion of the same Fv fragment from mammalian cells and *E. coli* we have shown that the latter is the system of choice for the production of large quantities of Fv protein. This is in contrast with results with intact IgG where high-level expression can be achieved with mammalian cells [39] but expression in *E. coli* yields only small amounts of protein [40]. It is of interest that the expression of individual CD4 Ig domains has not proved possible in CHO cells, although a polypeptide chain of two domains could be expressed to 25 mg/l [41].

Sedimentation results have demonstrated that the association of Vh and Vl to form Fv is concentration dependent. Associated Fv requires concentrations greater than 0.5 mg/ml, with complete dissociation taking place at concentrations below 0.2 mg/ml. As neither Vh or Vl is able to bind to the antigen when tested in isolation, assembled material is required for a signal in the antigen-binding assays. This suggests that assembly of Vh and Vl must be taking place at lower concentration in the conditions of the assay than seen in free solution. It is likely that this assembly at low concentration is assisted by the antigen itself. The interaction of Vh and Vl occurs through residues in both the framework regions and the complementarity-determining regions of the Fv [42]. Thus the stability of the Fv fragment is likely to vary from one antibody to another. The Fv fragment of McPC603 also dissociates readily into Vh and Vl, with an apparent dissociation constant in the order of 10 μM [38]. This is equivalent to approx. 0.2 mg/ml, similar to the concentrations at which B72.3 Fv dissociates. These workers also demonstrated apparently increased association in the presence of hapten by cross-linking studies. Vh and Vl of the D1.3 antibody have also been shown to be in dynamic equilibrium and readily exchanged [15].

Due to their small size Fv fragments are suitable for structural studies by both X-ray crystallography [43] and n.m.r. [10,44]. Analysis of the structure of the Fv of D1.3, both bound to antigen and unbound, has shown that Vl and Vh undergo a small rearrangement relative to each other on binding of antigen [3]. This induced fit to the antigen may explain the apparently more stably associated form of the Fv in the presence of the antigen. Crystals of a chimeric Fab of B72.3 have been obtained [45], and solution of the structure by X-ray diffraction recently achieved to 0.31 nm (3.1 Å) resolution [46]. Attempts to crystallize the Fv of B72.3 are now in progress.

Several approaches to the stabilization of Fvs have been taken by other workers, including the introduction of a disulphide bond between the Vh and Vl domains [38], and the production of single-chain Fv (scFv) fragments where Vh is joined to Vl through a peptide linker [38,47–49]. In these cases secretion of soluble scFv has often proved difficult with material produced as insoluble inclusion-body protein requiring solubilization and refolding to recover active material. ScFv molecules appear to

have a tendency to aggregate, possibly due to the exposure of hydrophobic residues at the Vh/Vl interface [38], which may limit their future use. In addition, the antigen-binding activity of scFv molecules produced has often been lower than that for the corresponding Fab fragment. However, examples of scFv which appear to retain full activity has been described recently [38,50].

Much interest in Fv fragments to date has centred on the use of Fvs raised against tumour-associated antigens for tumour imaging. In this application Fv fragments may have advantages over larger fragments or intact IgG. Fv fragments have been shown to penetrate further into the tumour mass than Fab or IgG and also to generate higher tumour/tissue (signal/noise) ratios [35]. The development of a high-yielding expression system for Fv in *E. coli* also opens the possibility of new applications of such fragments outside of healthcare where low-cost manufacture is required. These may include the use of Fv fragments in diagnostics, the food industry, agriculture and the cosmetics/toiletries industries.

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