

Three-dimensional structure of a human Fab with high affinity for tetanus toxoid¹

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Abstract

Background: The wide range of antibody specificity and affinity results from the differing shapes and chemical compositions of their binding sites. These shapes range from discrete grooves in antibodies elicited by linear oligomers of nucleotides and carbohydrates to shallow depressions or flat surfaces for accommodation of proteins, peptides and large organic compounds. **Objectives:** To determine the Fab structure of a high-affinity human antitoxin antibody. To explore structural features which enable the antibody to bind to intact tetanus toxoid, peptides derived from the sequence of the natural immunogen and antigenic mimics identified by combinatorial chemistry. To explain why this Fab shows a remarkable tendency to produce crystals consistently diffracting to d spacings of 1.7–1.8 Å. To use this information to engineer a strong tendency to crystallize into the design of other Fabs. **Study design:** The protein was crystallized in hanging or sitting drops by a microseeding technique in polyethylene glycol (PEG) 8000. Crystals were subjected to X-ray analysis and the three-dimensional structure of the Fab was determined by the molecular replacement method. Interactive computer graphics were employed to fit models to electron density maps, survey the structure in multiple views and discover the crystal packing motif of the protein. **Results:** Exceptionally large single crystals of this protein have been obtained, one measuring $5 \times 3 \times 2$ mm ($l \times w \times d$). The latter was cut into six irregular pieces, each retaining the features of the original in diffracting to high resolution (1.8 Å) with little decay in the X-ray beam. In an individual Fab, the active site is relatively flat and it seems likely that the protein antigen and derivative peptides are tightly held on the outer surface without significant penetration into the interior. There is no free space to accommodate even a dipeptide between V_H and V_L . One of the unique features of the B7-15A2 Fab is a large aliphatic ridge dominating the center of the active site. The CDR3 of the H chain contributes

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¹ The coordinates of B7-15A2 are available from the Brookhaven Protein Data Bank using ID code 1AQK.

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significantly to this ridge, as well as to adjoining regions projected to be important for the docking of the antigen. Both the ease of crystallization and the favorable diffraction properties are mainly attributable to the tight packing of the protein molecules in the crystal lattice. *Discussion:* The B7-15A2 active site provides a stable and well defined platform for high affinity docking of proteins, peptides and their mimotopes. The advantages for future developments are suggested by the analysis of the crystal properties. It should be possible to incorporate the features promoting crystallization, close packing and resistance to radiation damage into engineered human antibodies without altering the desired specificities and affinities of their active sites. © 1998 Elsevier Science B.V.

Keywords: Tetanus toxoid; Human Fab; Three-dimensional structure

1. Introduction

Tetanus toxin from *Clostridium tetani* causes spastic paralysis and convulsions by blocking the release of glycine, acetylcholine and other neurotransmitters through the prejunctional membranes of nerve cells [1–3]. After internalization of the toxin, which is a heterodimer of a heavy and light chain, the light chain acts as a zinc-dependent endopeptidase which selectively hydrolyzes synaptobrevin (between Glu 76 and Phe 77) and other target proteins. This cleavage is responsible for the blockade of neurotransmitter release [4]. Tetanus toxoid is prepared by denaturation of the toxin with formaldehyde. The toxoid is a very effective antigen for primary immunization against tetanus for adults and children two months of age and older. For individuals still requiring treatment, the contaminated tissues in the wounds are surgically removed (debridement) and the patient is given large doses of penicillin G and tetanus immunoglobulin from pools of human plasma. As in many infectious diseases, it should be feasible in the near future to replace the pooled immunoglobulin with monoclonal human antibodies produced in therapeutic quantities by recombinant technology.

In the present report we describe the structure of a prototypic human monoclonal IgG1- λ antibody, designated B7-15A2 [5]. B7-15A2 binds to the toxoid with high affinity (10^{10} M^{-1}) and also neutralizes the toxin, though somewhat weakly. Determination of the three-dimensional structure of the B7-15A2 Fab was performed as the first step in understanding the interactions between the antibody and the toxin. Amino acid sequences of CDR1 of the light chain and CDR3 of the heavy

chain were particularly intriguing because of their predicted influence on the size, shape and chemical properties of the antigen combining site. Among the projected structure-function relationships were expectations for a relatively large external surface suitable for docking of protein antigens. Cavities or crevices for end-on insertion of small ligands were neither expected nor found. For future studies B7-15A2 offered an unexpected benefit accruing from the ease with which high-quality crystals could be prepared. Detailed analyses of the protein packing in these crystals enabled us to identify features that can predispose other Fab constructs to crystallize in aqueous media.

2. Materials and methods

2.1. Preparation of the human B7-15A2 monoclonal antibody

A heterohybridoma cell line was used to prepare the antibody [5]. To establish the clone, human lymphoblastoid cells were fused to the human $\times$ mouse fusion partner K6H6/B5 and antibody producing clones were isolated by cloning at limiting dilution. Monoclonal antibodies were produced in 100 mg quantities in tissue culture flasks (conventional stationary cultures). The protein was precipitated from the supernate by the addition of ammonium sulfate to 50% saturation, isolated by centrifugation and purified by ion exchange chromatography on AB $\times$ 40 (J.T. Baker, Phillipsburg, NJ). B7-15A2 was adsorbed to the resin at pH 7.5 in 10 mM sodium phosphate and eluted at the same pH with 40 mM sodium phosphate buffer. A minor component was removed by rechromatog-

raphy on DEAE-Sephacel (Uppsala, Sweden) at pH 7.2 in 20 mM Tris.

2.2. Sequence analysis

Polymerase chain reaction was used to amplify cDNA derived from the hybridoma cell line. Amplified DNA was cloned into pEXmid3 and sequenced using the fmol DNA sequencing system (Promega, Madison, WI). These genes were assigned to their appropriate germ lines with the aid of the VBASE germline sequence directory provided by I.M. Tomlinson (MRC Unit of Molecular Biology, Cambridge, UK). Assignments were made with the FASTA algorithm in the Wisconsin sequence analysis package (version 8; Genetics Computer Group, Madison, WI).

2.3. Hydrolysis of the B7-15A2 IgG1 antibody with papain

Purified samples of the monoclonal antibody were cleaved into Fab and Fc fragments with papain at pH 6.8. The initial concentration of antibody was 21.5 mg/ml of 50 mM bis(Tris), 50 mM Mops, 0.15 M NaCl and 1 mM NaN₃. Mercuripapain (Sigma, St. Louis, MO) was activated with EDTA and β -mercaptoethanol and added to the protein in a ratio of 1 mg enzyme to 42 mg antibody. Hydrolysis was carried out at 4°C for 15.5 h, after which the reaction was terminated by the addition of 3.0 M iodoacetate.

Aggregates, undigested IgG1 protein and large fragments were removed from the reaction mixture by gel filtration on a 5 × 100 cm column of Sephacryl S200 equilibrated at 22°C with 20 mM Tris, 0.15 M NaCl, pH 8.0. Fractions containing the Fab were collected at 4°C, pooled, placed in a dialysis membrane and concentrated by placing the dialysis bag over polyethylene glycol (PEG) 20 000 powder. To separate the Fab from residual Fc, the solution was loaded onto a 2 × 20 cm column of CM-52 (carboxymethyl cellulose), equilibrated with 25 mM histidine, pH 5.6. The column was successively washed with this buffer and pH 7, 40 mM Mops, both containing 1 mM NaN₃. The Fab was eluted at pH 8.5 with 40 mM Tris and 1 mM NaN₃.

2.4. Crystallization of B7-15A2 Fab

Prior to crystallization by a microseeding technique [6] in PEG 8000, aliquots of the Fab were concentrated to 100–150 mg/ml by vacuum dialysis. Crystals spontaneously appeared in small droplets (5 μ l) of the protein solution made 7–8% (w/v) in PEG 8000. Seeds were prepared by washing these crystals with 13.2% PEG 8000 in 1 mM NaN₃. This suspension was diluted 1:10 000 to obtain a small number of seeds. Crystals suitable for X-ray diffraction were obtained by adding 1–3 seeds to a solution containing 93 mg of Fab per ml of 31 mM Tris, pH 8.5, 7.3% (w/v) PEG 8000, and 0.8 mM NaN₃. In two days these crystals grew to > 0.2 mm in length, and it was not unusual to obtain crystals 2.0 × 1.5 × 1.0 mm ($l \times w \times d$) in 1 week. The crystal used for X-ray analysis was 5 × 3 × 2 mm in size. Six fragments of irregular shape and size were produced by cutting this crystal with a small scalpel. Each fragment diffracted X-rays to at least 1.8 Å resolution with very little decay in the X-ray beam.

2.5. Collection of X-ray diffraction data

X-ray data were collected at 13°C with a Siemens × 1000 area detector interfaced to a rotating anode generator (operated at 40 kV and 80 mA). Diffracted rays were directed through a helium path to the detector, which was set 18 cm from the crystal. Data were processed on a Silicon Graphics Workstation using the XENGEN software package [7]. Data to 1.8 Å resolution were used for refinement.

2.6. Determination of the three-dimensional structure of the B7-15A2 Fab

The structure of the B7-15A2 Fab was solved by molecular replacement methods [8–10]. Starting models for these calculations were chosen from the Brookhaven Protein Data Bank [11,12]. Whole Fab molecules, as well as V_L–V_H and C_L–C_H1 domain pairs, were evaluated for all candidates. The human Kol Fab [13], derived from an IgG1- λ myeloma protein, produced the highest peaks in the rotation function studies,

which were conducted with the MERLOT program package [9]. Translation functions for placing the models of the intact Fab and the domain pairs in the B7-15A2 unit cell were calculated with the program RTMAP (Joseph He, as quoted in [14]).

The preliminary model obtained by molecular replacement was subjected to rigid body refinement with a program in the X-PLOR package [15]. This was followed by positional (Jack-Levitt) refinement and simulated annealing with X-PLOR programs. Fo-Fc OMIT [16] maps were then calculated for this partially refined model. Kol sequences were replaced with their B7-15A2 equivalents and residues were fitted to the OMIT electron density maps on a Silicon Graphics Personal IRIS workstation with the program TURBO-FRODO [17]. The resulting B7-15A2 model was re-introduced into X-PLOR for positional refinement. Simulated annealing with X-PLOR was alternated with interactive model building and positional refinement until the fluctuations in the atomic coordinates were minimal.

Considering the quality and resolution of the data, we felt that the *R* factor of 0.22 was too high at this stage of the refinement. Adjustments in the model, particularly in CDR3 of the L chain, improvements in the stereochemistry of the loops, and a change in the refinement program to TNT [18] led to a rapid reduction of the *R* factor to its present value of 0.185. The structural analysis was completed by *B* factor refinement and addition of 275 ordered water molecules.

2.7. Binding of peptides to B7-15A2

Combinatorial chemistry [19] (Pepscan) was used to identify productive ligands to B7-15A2. A second approach based on phage display yielded peptides of 22 residues that were tested for binding to B7-15A2 (W. Huse, IXSYS, personal communication). Two peptides were selected from these binding trials, synthesized in mg quantities by Chiron Mimotopes (Melbourne, Australia) and kindly supplied to us by Gordon Tribbick. The first contained the C-terminal 15 residues of a 22-mer from the phage displayed peptide library: Ac-Phe-Gln-Ser-Tyr-Trp-His-Glu-Ile-Leu-Gly-

His-Ser-Ser-Tyr-Leu-NH₂. This sequence was taken from the N-terminal region of tetanus toxin with a signature sequence of Cys-Cys-Tyr. For ease of synthesis of the peptide, serine was substituted for the two half-cystines. The second was an octapeptide with the sequence Ac-Ile-Trp-Ile-Val-Pro-Glu-Arg-Tyr-NH₂. Both peptides were 'mimotopes', designed to mimic but not duplicate an actual epitope on the surface of the antigen.

Dissociation constants (*K_D*) for the binding of each peptide to the B7-15A2 Fab were determined by the spectrophotometric method of Droupadi et al. [20]. This procedure is based on *n*- π and π - π^* interactions of ligand and protein in the formation of a complex and have been used by us to measure dissociation constants over a range of 10^{-2} – 10^{-10} M.

Attempts have been made to diffuse each of these two peptides into pre-existing crystals of B7-15A2 Fab by well established procedures [21–23]. However, analyses of the crystal packing (Section 3) cast doubt on the prospects for success because of the limited access to the active site. Cocrystallization of the Fab with each peptide appears to be a more sensible strategy and such experiments have been initiated. Small crystals have recently been obtained.

3. Results

3.1. Amino acid sequences of the L and H chains of the B7-15A2 Fab

Sequences of B7-15A2 V_L and V_H are listed in Fig. 1. Two other sequences of human λ -type proteins, Mcg [24] and Kol [25], are included with that of the B7-15A2 L chain. V_H sequences of the Kol IgG1 [26] and the Pot IgM immunoglobulins [14] are listed for comparisons of H chains. Since the C domains were not subjected to sequence analysis, the residues presented for B7-15A2 are taken from a standard C_L2 sequence (Swiss-Prot entry P01842) and the Kol C_H1 domains. The numbering system of Kabat et al. is used both to compensate for insertions or deletions and to facilitate comparisons of different antibodies [27].

HEAVY CHAIN				
V _H DOMAIN				
	FR1	CDR1	FR2	
	10	20	30	40
	:	:	:	:
B7-15A2	ZVQLVESGGGVVQPG	SLRLSCAASGFTFNN	NYAIH	WVRQAPGK
Kol		SS...I.SS..	MY	
Pot	E.H.L....NL....G.....	IFVMS		
	FR2	CDR2	FR3	
	50	60	70	80
	:	a	:	abc
B7-15A2	GLEWVAF	FISYDGSKNYYADSVKGR	FTISRDN	SKNTLFLQMN
Kol		I.WD...DQH		D..
Pot		SGVFGS.GNTD...A	T.....	Y.....
	FR3	CDR3	FR4	
	90	100	110	
	:	abcdefghi	:	
B7-15A2	RPEDTAIYYCARVLF	QQLVLY---	APFDI	WGQGTMTV
Kol	GV.F...	DGGHGFCS	SASCFGP.Y	P.....
Pot	.A.....	KHRVSYVLTG-----	S	L.....
LIGHT CHAIN				
V _L DOMAIN				
	FR1	CDR1		
	10	20	30	
	:	:	abc	:
B7-15A2	ZNVLTPPPS-VSGAPG	QQRVTISCT	TGSNSNIGAGFTV	HW
Mcg	.SA.....-A..SL..S.....	TS.DV.GYNY.S		
Kol	.S.....-A..T.....	S.TS..-IGSS..N		
	FR2	CDR2	FR3	
	40	50	60	70
	:	:	:	:
B7-15A2	YQHLPGTAPKLLIF	ANTNRPS	GVDPDRFSGSKSGT	SASL
Mcg	..QHA.K...VI.YE	VNK	NT...	
Kol	..Q...M.....	YRDAM	A....	
	FR3	CDR3	FR4	
	80	90	100	
	:	:	ab	:
			a	
B7-15A2	AITGLQAEDEADYYC	QSYDSSL	S-ARFGG	TRLTVLG
Mcg	TVS.....	S..EG.DN-FV	..T..KV....	
Kol	..G...S...T....	AAW.V..NAYV	..T..KV....	

Fig. 1. Amino acid sequences of the B7-15A2 V_L and V_H domains. For comparative purposes, the residues are numbered according to the system of Kabat et al. [27], with appropriate insertions and deletions identified by lower case letters and blank spaces, respectively. Sequences of the C_L and C_{H1} domains were not determined experimentally, but were assumed to be closely similar to those of a standard C_{L2} domain (Swiss-Prot entry P01842) and the C_{H1} sequence of the Kol IgG1 immunoglobulin [26].

CDR1 in the B7-15A2 L chain (LCDR1) has a conformation like those in the λ chains of New [28] and Kol [13], but the sequence is one residue longer than in Kol. LCDR2 is consistent with those of other λ chains in length and structure. LCDR3 has the same length as in Mcg [24] but is one residue shorter than in Kol and one residue longer than in New. It is different in sequence and conformation from all three.

It has been proposed that the CDRs of λ chains follow a common pattern of canonical structures [29]. However, X-ray analyses of the Mcg dimer [30] indicated that LCDR1 regions with identical amino acid sequences assumed different conformations in the two monomers. Subsequently, the LCDR1s of the human Hil [31] and the murine Se155-4 Fabs [32] were also shown to be exceptions to this generalization. LCDR2 regions generally conform to an expected conformation pattern. As illustrated by the B7-15A2 results, the LCDR3 structures are beginning to show deviations even in the limited number of λ chain structures currently available.

The H chain of B7-15A2 contains sequences specifying canonical structures of type 1 for HCDR1 and type 3 for HCDR2 [29,33]. The product of three genes (V_H , D and J_H), HCDR3 exhibits extremely high variability. It is therefore considered too hypervariable in structure to assign to a canonical niche. In B7-15A2, HCDR3 is two residues longer than in the human Pot Fv from a human IgM cryoglobulin [14] and three residues longer than in the murine NC41 Fab [34], specific for influenza neuraminidase. Although the B7-15A2 HCDR3 is relatively large, it is exceeded in length by at least three other Fabs, from the murine anti-cyclosporin antibody R454511 [35], the human anti-GP41 antibody 3D6 [36], and the human Kol myeloma protein [13]. Unlike its counterpart in Kol, B7-15A2 HCDR3 is not constrained by a disulfide bond cross-linking the loop at the region where it enters external solvent.

3.2. Space group and unit cell dimensions of the Fab crystals

The space group was monoclinic $P2_1$, with $a = 40.7$ Å, $b = 78.6$ Å, $c = 74.6$ Å and $\beta = 104.3^\circ$.

Table 1
Statistics for data collection

Resolution (Å)	1.84
Theoretical no. of reflections	39 263
Observed reflections ($I > 2(I)$)	32 494
Completeness	82.7%
R_{merge}	4.6%
Average redundancy	2.5

The volume of the unit cell was $231\,077$ Å³ or $115\,034$ Å³ per asymmetric unit (one Fab molecule). Using Matthews' approximation, we calculated that the protein occupied 50% of the volume of the unit cell, a value well within the normal range [37].

3.3. X-ray diffraction data and crystallographic refinement

Statistics for the collection of X-ray diffraction data for B7-15A2 are given in Table 1. Between 20 and 1.84 Å resolution, the data set was 83% complete (32 494 unique reflections of the theoretical total of 39 263 were observed). This value was $> 90\%$ to 2.6 Å and 80% for the shell extending from 2.11 to 1.96 Å. Only 102 of approximately 82 000 reflections were found to have intensities (I) $< 2\sigma(I)$. Even in the last shell (1.84–1.86 Å) the average value for $\sigma(I)$ was 6.37. For all data, the R_{merge} value was 4.59%, and the average number of measurements for each reflection was 2.5.

Table 2
Statistics for crystallographic refinement

No. of protein atoms	3313
No. of water molecules	275
No. of reflections	32 494
Resolution range (Å)	1.84–20.0
$R = F_o - F_c / F_o$	0.185
R.M.S. deviations from ideal values	
Bond lengths (Å)	0.017
Bond angles	2.8°
Main chain torsion angles in Phi/Psi plots	
Most favored regions	84.7%
Additionally allowed	13.4%
Generously allowed	1.1%
Disallowed	0.8%

Refinement statistics are presented in Table 2. For 6–1.84 data, the structure has an *R*-factor of 0.185, with root-mean-square (R.M.S.) deviations of 0.017 Å in bond lengths and 2.8° in bond angles. The mean error in coordinates was estimated to be 0.25 Å [38]. Using Wilson Statistics, a value of 20.0 Å² was obtained for the average temperature (*B*) factor. As expected, individual temperature factors varied in accordance with the quality of electron density. For example, disordered polypeptide backbone and side chain constituents at the carboxyl ends of the chains and in one helical loop of the H chain (Section 3.4) exhibited significantly higher *B* values than those for the rest of the Fab molecule.

In comparison with other analyses of proteins at the same resolution, main chain parameters evaluated with the program PROCHECK [39] were average or better with regard to Ramachandran plot quality assessment, peptide bond planarity, measure of bad non-bonded interactions, alpha carbon tetrahedral distortion, zeta angle standard deviation, and hydrogen bond energies. Side chain parameters were also found in the expected range. In the Ramachandran plot (not shown), 86.1% of the residues were located in the most favored regions of the ϕ , ψ plot and 12.9% were in additional allowed regions. Two residues on L chain loops (Asn L51 and Asp L151) were found in disallowed regions. *cis*-Peptide bonds were detected at three positions occupied by proline, one in the L chain (L141) and two in the H chain (H149 and H151). L141 and H149 are structural equivalents in the two chains.

3.4. Electron density

The quality of the electron density map is illustrated for residues L31–L38 in Fig. 2. Most of the other regions were also well represented by modules of electron density (ρ) at levels at least as high as 1.5–2.0 $\sigma(\rho)$. Weaker, but still continuous electron density (1.0 $\sigma(\rho)$) was obtained for two short segments, L1–L3 (N-terminal region) and L91–L93 (CDR3). Fragmented electron density was found in $F_o - F_c$ OMIT maps [16] for the C-terminal regions of both L (L212–L215) and H chains (H230–L233) and for the first loop in the

C_H1 domain (H127–H136). These segments are often troublesome regions, as evidenced by varying degrees of disorder cited for at least nine other Fab structures in the Brookhaven Protein Data Bank.

Ordered water molecules generally appeared as well defined spheres of electron density. Of the 275 assigned to density modules within 5 Å of the protein surface, 193 were within hydrogen bond distance (< 3.2 Å) of protein oxygen and nitrogen atoms and 38 were in networks with other water molecules. Many water molecules were in contact with more than one protein atom. There were examples of water bridging two protein polar groups and of networks of three or more solvent molecules between symmetry-related Fab units.

Of those solvent molecules near the polypeptide backbone, 65% were associated with carbonyl oxygens and 35% with amide groups. Interactions with side chain oxygen atoms in hydroxyl (serine, threonine and tyrosine) and carbonyl groups (aspartic and glutamic acids, asparagine and glutamine) outnumbered those with nitrogen atoms (arginine, lysine, histidine and tryptophan) by a ratio of 71 to 29%. Few water molecules were found along the internal opposing surfaces of V_L and V_H because the side chains occupied practically all of the available space (Section 3.9).

3.5. Diffusion versus cocrystallization to produce ligand-protein complexes in crystals

Diffusion of Ac-Ile-Trp-Ile-Val-Pro-Glu-Arg-Tyr-NH₂ or Ac-Phe-Gln-Ser-Tyr-Trp-His-Glu-Ile-Leu-Gly-His-Ser-Ser-Tyr-Leu-NH₂ into pre-existing crystals of the B7-15A2 Fab did not result in overt physical damage or interfere significantly in the ability of the crystals to diffract X-rays. There were no losses in resolution, widening of X-ray reflections, or decay increases over time. However, the presence of bound peptide could not be detected by difference Fourier analysis. Accessibility is the key attribute and it seems likely that appropriate solvent channels are not available in the crystals for direct transport of the ligands to the Fab active sites.

Cocrystallization is the obvious alternative and preliminary results are very encouraging. A mix-

ture of Fab and 1.1 molar quantities of the 15-mer produced a shower of small crystals overnight when treated with PEG 8000 at 20°C. Conditions are now being optimized to prepare large single crystals for X-ray analysis.

3.6. Binding of representative peptides to B7-15A2 in solution

Because of the size (150 kDa) of the intact toxoid, the preparation of a crystalline complex with B7-15A2 is a daunting prospect. Moreover, no large toxin fragment produced by recombinant technology has as yet been demonstrated to have antigenic reactivity for the antibody. In contrast, there is little difficulty in finding smaller peptides that bind with reasonable affinity. For instance, the K_D values for the 8- and 15-mers

discussed above were 1.4×10^{-6} and 8.5×10^{-9} M.

The dipeptide Asp-Phe methyl ester (aspartame) did not show any affinity for B7-15A2 in solution, even at the lower limit (10^{-2} M) of the detection method (Leif Hanson, unpublished work). In the early days of combinatorial chemistry, Gordon Tribbick, Mario Geysen and Allen Edmundson introduced dipeptides as probes for antibodies with cavity-type binding sites [23,40]. Currently, Asp-Phe methyl ester is one of our favorites [41–43] to map crevice- or cavity-type sites in murine antibodies (e.g. NC6.8) and human Bence-Jones dimers (e.g. Mcg). We interpreted the failure of this peptide to bind as a probable indication that the active site of B7-15A2 is tightly sealed against the entry of small ligands. The three-dimensional structure of the binding site

Fig. 2. Three-dimensional 'cage' electron density for residues L31 to L38 of the light chain, with skeletal models superimposed. From the top, the amino acid sequence is Phe-Thr-Val-His-Trp-Tyr-Gln-His. Side chains on the left face the heavy chain and the ones on the right are directed towards the interior of the V_L domain.

Fig. 3. Model of the B7-15A2 Fab constructed with the program MOLMOL [44]. The L chain is on the right and the H chain on the left. V domains are located in the top half of the molecule and the C domains are in the lower half. Tubular backbone segments are colored yellow in the L chain and steel blue in the H chain. The 5-stranded antiparallel β -pleated sheets facing each other are represented by magenta arrows in V_L and white arrows in V_H . Four-chain β -pleated sheets on the outer surfaces are colored cyan in V_L and red in V_H . The three CDR loops in each chain are marked with numbers in the appropriate colors at the upper tip of the molecule. In the C domains the 4-stranded β -pleated sheets are colored cyan (L chain) and red (H chain), as in the V domains. However, here they form the interacting surfaces while the 3-chain layers (magenta and white arrows) are oriented towards the external solvent. Note that the Fab has an overall extended conformation; the 'elbow bend' angle between the V and C pseudodyads is 165°. Side chains are tightly packed in the space between the two V domains. Even potential ligands as small as dipeptides are excluded from this region. Tetanus toxoid, the protein antigen used to elicit B7-15A2, and cross reacting peptides are probably accommodated on the relatively flat outer surfaces of the active site.

Fig. 4. Stereo diagram of selected residues from the central portion of the active site. Around the upper right perimeter from about 12 to 4 o'clock are components of CDR3, CDR1 and CDR2 of the light chain. At the top is the Asp-Ser-Ser-Leu-Ser segment of LCDR3 (residues L92–L95a). Gly-Phe (L30–L31 of LCDR1) is located at the upper right. Phe-Ala-Asn-Thr-Asn-Arg (L49–L54 of LFR2 and LCDR2) is found in the lower right. Heavy chain CDR constituents occupy the space counterclockwise from 11 to 6 o'clock. In the upper left is an HCDR2 segment Asp-Ser-Val-Lys (H61–H64). A second HCDR2 fragment, Tyr-Asp-Gly-Ser-Lys (H52a–H56), is located in the middle left section. Just below Tyr H52a is Tyr H32 of HCDR1. In the middle of the site above and to the right of Tyr H32 is the HCDR3 segment Leu-Val-Leu-Tyr-Ala-Pro-Phe (H100–H100i). Leu L95, Leu H100 and Leu H100b form an aliphatic ridge in the center of the site. The combination of Phe L31 and Tyr H100c block access to the interior and add to the aromatic character of the site (Phe L31 and L49; Tyr H32, H52a and H100c). Along the upper left and top, the periphery is laced with polar side chains (Asp H53 and H61; Ser H55, H62, L93, L94 and L95a; and Lys H56 and H64).

Fig. 5. Stereo diagram of one set of interactions blocking access of potential ligands to the V_L – V_H interface. On the right is the same L chain strand as that shown in Fig. 2, encompassing residues L31–L38 (last four residues of LCDR1 and first four residues of LFR2). H chain components on the left include residues H100f–H100i (HCDR3) at the top and Gln H39 (HFR2) at the bottom. Note that the seal starts with interactions between Phe L31 and Tyr H100c at the top, and continues with the semi-stacking of His L34 and Pro H100h. Other interactions occur between Tyr L36 and HCDR3 (both Pro H100h and Phe H100i). At the bottom this zipper-like closure is completed by the juxtaposition of His L38 and Gln H39. Relative to the human Mcg L chain [24], this closure is facilitated by the replacement of three aliphatic residues by aromatic rings: Phe L31 for Asn, His L34 for Ser and His L38 for Gln (Fig. 1).

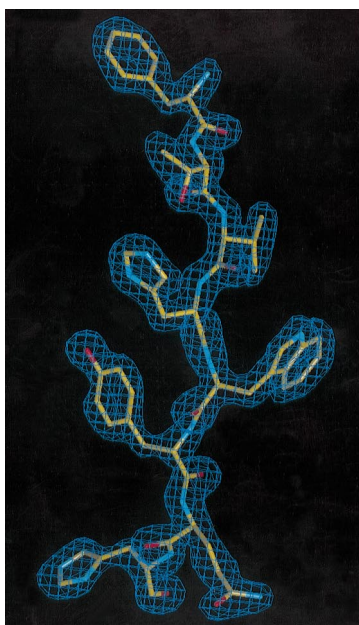


Fig. 2



Fig. 3

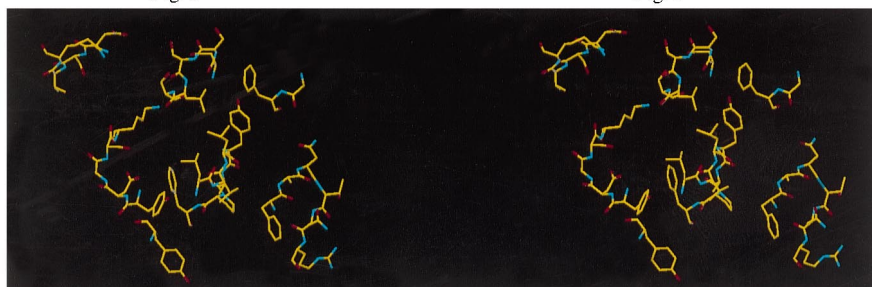


Fig. 4

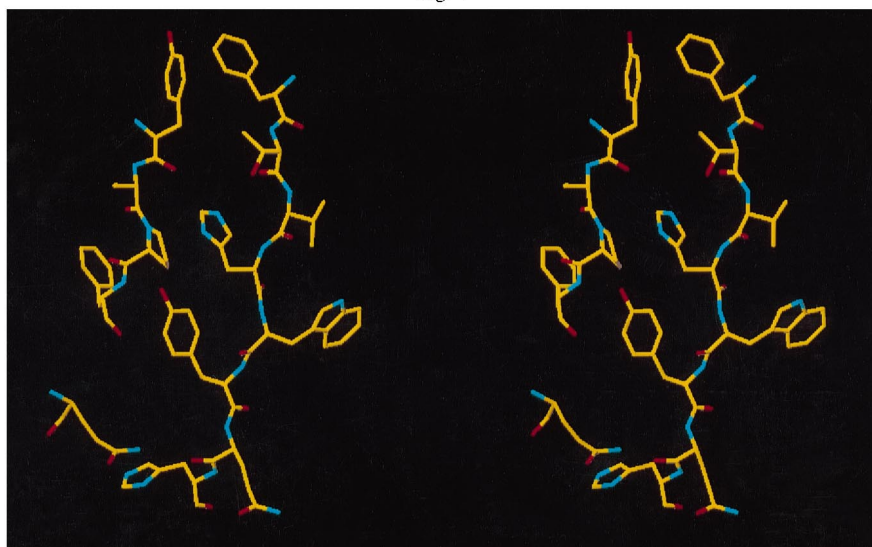


Fig. 5

Figs. 2–5.

was compatible with this view (see following sections).

3.7. General structural features of the Fab and its components

A model of the B7-15A2 Fab constructed with the program MOLMOL [44] is presented in Fig. 3. In overall appearance, this Fab bears a strong resemblance to other human and murine antibody fragments and light chain dimers [45]. As in all of these molecules, the B7-15A2 Fab has pseudo-two-fold axes of symmetry relating both the V_L-V_H (169°) and C_L-C_H1 pairs (170°) of domains. When compared with the Fab portions of intact human immunoglobulins with λ -type L chains and γ_1 H chains, B7-15A2 more closely approximates Kol [46] than Mcg [47,48]. B7-15A2 and Kol have extended structures in which the elbow bend angles between the V and C pseudodyads approach 180° (165° for B7-15A2 and 174° for Kol), while the Mcg Fab region has a pronounced elbow bend of 118° .

Indicators of extension and flexion can easily be calculated if cylindrical envelopes are constructed around the domains [30,49]. In contrast to the Mcg IgG1, the B7-15A2 L chain is less extended than the H chain, as evidenced by the angles subtended by the long axes of the V and C cylinders: 86.0° for the L chain and 92.5° for the H chain. The corresponding values for the Mcg IgG1 protein are 108° for the L chain and only 30° for the H chain.

3.8. Description of the binding site of B7-15A2

The putative active site of B7-15A2 is shown in Fig. 4. Polypeptide backbone segments of the CDR loops of the H chains are generally swept away from the L chain CDRs and flattened against the globular body of the V_H domain. The net effect is to produce a broad binding surface suitable for the docking of large antigens like tetanus toxoid. HCDR3, which is located in the center of the site, contains a series of hydrophobic side chains (Leu H100, Val H100a, Leu H100b) projecting directly into the solvent. This unusual aliphatic ridge extends to Leu L95 from LCDR3.

Several prominent aromatic residues surround the central aliphatic region: Tyr H100c and Phe H100i from HCDR3; Tyr H52a from HCDR2; and Phe L31 from LCDR1. Framework residue Phe L49 helps fill the space between LCDR2 and HCDR3 and is also a potential contact residue. Charged residues are located at the periphery of the site: Asp H53, Lys H56, Asp H61 and Lys H64 in HCDR2 and Arg L54 in LCDR2. Asp L92 in LCDR3 is nearby, but is probably not available for binding without significant conformational adjustments in the binding site.

3.9. Sealing of the active site of B7-15A2 to the entry of small ligands

3.9.1. Blocking feature 1: three aromatic-for-aliphatic substitutions in the L chain

In the large conical binding cavity of the Mcg λ -chain dimer, the entrance is flanked by two tyrosine side chains symmetrically placed on opposing CDR1 loops [50]. These residues correspond to Thr L32 and Ala H33 in B7-15A2 (Fig. 1). They are not accessible even to solvent because of a structural canopy formed by LCDR1 and HCDR3. These interactions are illustrated in Fig. 5.

Major contributing factors to this closure are three substitutions of aromatic for aliphatic side chains in CDR1 of the B7-15A2 L chain, relative to Mcg. The first is an L31 Phe for Asn substitution occurring just above Thr L32 in Fig. 2. HCDR3, which is much larger than its counterpart in the Mcg dimer, consumes the remaining space that would otherwise be available in this region. Tyr H100c on HCDR3 makes van der Waal's contact with Phe L31 to close the canopy. Throughout the text, contacts are defined by distance criteria in which two atoms are separated by less than $4 \text{ \AA}$ (the sum of the atomic radii of two carbon atoms, plus a tolerance of $0.4 \text{ \AA}$).

A second substitution, His L34 for Ser, in Mcg results in eight additional van der Waals contacts with HCDR3 residues Tyr H100c, Ala H100g and Pro H100h. At this juncture, LCDR1 technically ends but the V_L-V_H zipper continues with LFR2 residues Tyr L36 and His L38. The latter is the third example of an aromatic-for-aliphatic substi-

tution (His for Gln in Mcg). Tyr L36 participates in 12 interactions with HCDR3 components, Pro H100h and Phe H100i. Its phenolic hydroxyl group forms a hydrogen bond (3.1 Å) with the backbone amide group of Phe H100i. His L38 is directed toward Gln H39 to complete this part of the V_L – V_H seal.

3.9.2. Blocking feature 2: contacts of LFR2 with HFR2, 3 and 4 and with HCDR3

As the LFR2 strand loops back (ascending arm) it again comes into close contact (18 interatomic interactions) with V_H components (not shown). Thr L42, Ala L43, Pro L44, Leu L46, and Phe L49 successively encounter Tyr H91 (HFR3), Trp H103 (HFR4), Gly H104 (HFR4), Leu H45 (HFR2), Phe H100i (HCDR3) and Pro H100h (HCDR3). As mentioned above, Phe L49 is especially prominent in filling the hole between LCDR2 and HCDR3.

In the Mcg dimer two of LFR2 residues (L43 and L44) listed above are constituents of a deep binding pocket and two are ligand contact residues (L46 and L49) in the main binding cavity. Their equivalents are all shielded from both ligand and solvent by the H chain constituents of B7-15A2.

3.9.3. Blocking feature 3: burial of LCDR3 and adjoining segments

Completion of the seal was made by engagement of the L chain loop beginning with Tyr L87 in LFR3 and continuing through the LCDR3 loop to Phe L98 in LFR4 (not shown). Tyr L87 was immobilized and buried by eight interactions with Gln H39, Gly H44 and Leu H45, all from HFR2. On the ascending arm of LCDR3, the side chains facing V_H were interlocked with those from HCDR3 (17 contacts). Gln L89 interacted with Phe H100i, Tyr L91 impinged mainly on backbone atoms of Tyr H100c and Ala H100g and Ser L93 encroached on the side chain of Tyr H100c.

On the descending arm of LCDR3, the coverage was even more comprehensive. Ser L94 made 14 contacts with Lys H56 and Tyr H58 (HCDR2), including a hydrogen bond between the L94 backbone carbonyl oxygen and the ϵ -amino group of Lys H56. Backbone atoms of Leu L95 also

touched Tyr H58, as well as Trp H47 (HFR2). The Leu L95 side chain was locked into position by nine contacts with Phe H50 (HCDR2). Both the backbone and side chain atoms of Ser L95a and Ala L96 were thoroughly covered by Trp H47 (15 contacts). These interactions of V_L with V_H were terminated by six contacts of Phe L98 (LFR4) with Leu H45 (FR2) and Phe H100i (HCDR3).

All of the L chain constituents (or equivalents) mentioned in the last paragraph have been identified as contact residues for one or more ligands bound to the Mcg dimer. In toto, there seems to be no possible access for even the smallest of these ligands to penetrate the border between the V_L and V_H domains of B7-15A2.

3.10. Packing interactions of the B7-15A2 Fab molecules in the space group $P2_1$

The moderately dense packing of the protein in the crystal lattice ($V_{\text{PROT}} = 50\%$) was favorable for prospects of both high quality and high resolution diffraction. Both prospects were realized in this study. Close packing of protein molecules was attributable to five major sets of intermolecular interactions: (1) side-by-side stacking of Fab molecules like spoons; (2) antiparallel alignment of C_L and C_{H1} domains in symmetry related molecules, mediated through the formation of cross-molecule β -pleated sheets; (3) head-to-tail interactions of CDR1 of V_L with the final turn of C_{H1} ; (4) contacts of CDR1 and FR3 of V_L with the first turn of V_H ; and (5) interactions of loops of V_L with side chains of CDR2 and FR3 in V_H from neighboring molecules. Sets 1 and 2 were dominant in dictating the packing and will be considered here in detail.

3.10.1. Side-by-side crystal packing

This type of packing, which is illustrated in Fig. 6, is mainly attributable to the extended conformation of the B7-15A2 Fab and the shape complementarity of its various components. The C_L – C_{H1} domain pair fits beautifully into the concave space between the V and C modules of an adjacent Fab molecule. With few exceptions the interactions across this space are restricted to

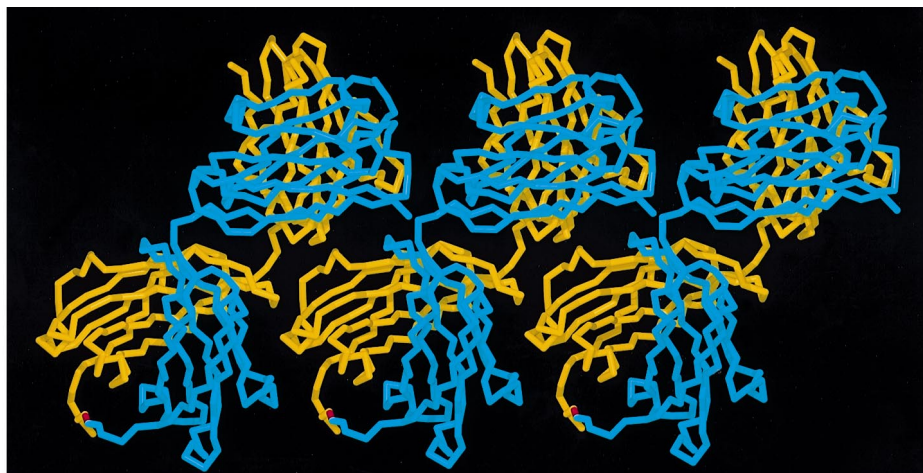


Fig.6

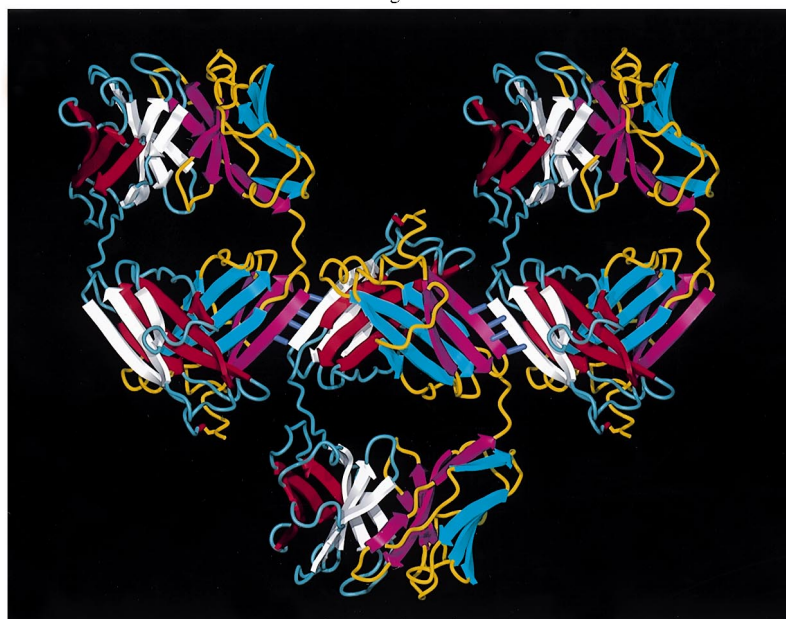


Fig.7



Fig.8

Figs. 6–8.

juxtaposed C_L domains (see opposing yellow loops in Fig. 6), while the upper contacts are limited to V_H constituents (blue). The total number of interatomic contacts $< 4 \text{ \AA}$ is 80. For the discussion below, we will focus on the central molecule of the three shown in Fig. 6.

Residues L150–L153 (Ala-Asp-Ser-Ser) of the broad yellow loop jutting out to the left participate in 28 interactions with the backbone and side chain atoms of the sharp, twisted loop of residues L168–L170 (Ser-Asn-Asn) protruding toward it from the immediately adjacent molecule. These interactions are repeated in reverse on the right side: i.e. the L168–L170 residues are supplied by the central Fab and the L150–L153 loop is located on the next molecule. In the V_H portion of the central molecule, the wide blue loop at the far left of the domain, containing residues H83–H85 (Arg-Pro-Glu), makes 23 contacts with a tripeptide segment of residues H26–H28 (Gly-Phe-Thr) in the opposite Fab. These two loops are further held in close proximity by two hydrogen bonds between the side chain carboxyl oxygens OE1 and OE2 of Glu H85 with the backbone and side chain of Thr H28. Again these stabilizing interactions are duplicated on the other side of the central molecule.

From a practical view, the beginning of the H chain was easy to identify because the N-terminal tripeptide segment was immobilized by crystal packing interactions. In Fig. 6, this segment is seen as a blue rod extending downward from the lower right portion of V_H . The pyrrolidone carboxylic acid (PCA, or cyclized glutamine) residue

in position 1 and the side chain of Gln H3 of the central molecule make 16 contacts with the H178–H181 (Leu-Gln-Ser-Ser) loop protruding upward and to the left from C_{H1} in the adjacent Fab.

3.10.2. Packing dictated by the formation of antiparallel β -pleated sheets between C_L and C_{H1} of symmetry-related molecules

The ‘immunoglobulin fold’ has been characterized as a ‘ β -barrel’ or a ‘ β -sandwich’ because of the propensity of its amino acid sequence to produce strands of antiparallel β -pleated sheets. This tendency is carried over into the free surfaces along the outer strands, which are already oriented in favorable positions by hydrogen bonding to an adjacent segment on their inner surfaces. The N-terminal segment of V_L (first strand of the 4-chain β -pleated sheet, designated 4-1) and the last strand (3-3) in the 3-chain β -pleated sheets of C_L and C_{H1} are particularly accessible to solvent and therefore available for docking of sizable structures.

As illustrated in Fig. 7, a second dominant packing motif (compare Fig. 6) in the crystallization of the B7-15A2 Fab consists of bilateral intermolecular hydrogen bonding and ribbon formation. The symmetry operation (characteristic of the $P2_1$ space group) relating adjacent molecules is a two-fold rotation about the y -axis and a translation of one-half the unit cell dimension. On one side of an Fab (e.g. second from the right), backbone atoms of the 3-3 strand of C_L make four hydrogen bonds with the 3-3 segment in C_{H1} from

Fig. 6. Crystal packing diagram of three adjacent Fab α -carbon models translated by one unit cell along the a -axis. The L chain is colored yellow and the H chain blue. V_L – V_H pairs are located at the top of the diagram and C_L – C_{H1} domains are at the bottom. Note how well the C_L – C_{H1} modules fit into the hollows between the V and C pairs of adjacent Fab molecules. An analogy is the stacking of spoons. The red segment indicates a disulfide bridge.

Fig. 7. Crystal packing diagram of three Fabs, each related to the next by a 2_1 axis (a rotation of 180° about the vertical axis and a translation of one-half of the unit cell dimension). The Fabs are represented by models with the same color patterns as those used in Fig. 3. On one side of each molecule the polypeptide backbone of the last strand in C_L (magenta arrow) is hydrogen bonded to its counterpart in C_{H1} (white arrow) of an adjacent, but antiparallel Fab. The opposite arrangement occurs on the other side of the Fab: i.e. the C_{H1} is hydrogen bonded to C_L in the next molecule. These hydrogen bonds are schematically shown as small rods in the intermolecular spaces. Thus, each three-chain layer joins its partner to form a six-stranded, antiparallel β -pleated sheet spanning two Fabs. The net effect is to align the Fab molecules into a ribbon of indefinite length in the crystal lattice.

Fig. 8. A stereo diagram of three Fab molecules in the antiparallel arrangement shown in Fig. 7. Note how the depth perception in the stereo version leads to a better understanding of the formation of a cross-molecule β -pleated sheet (e.g. the strong twist in the 6-stranded sheet).

a symmetry-related molecule (Fab at far right). The hydrogen bonds are formed between: (1) the carbonyl oxygen (CO) of Gly L200 and the backbone amide group (NH) of Asp H220; (2) the NH of Thr L204 and the CO of Lys H218; (3) the CO of Thr L204 and the backbone NH of Lys H218; and (4) the NH of Glu L206 and the backbone CO of Asn H216. The reciprocal participation of the NH and CO atoms of Thr L204 and Lys H218 is an identifying characteristic of antiparallel β -pleated sheets. On the opposite side of the selected Fab, this hydrogen bonding pattern is repeated between C_H1 and C_L of the next Fab in the series (third from the right). A stereo version of Fig. 7 is presented in Fig. 8 to provide a better understanding of the spatial relationships in the packing of adjacent Fab molecules in the lattice. Note that the newly formed β -pleated sheets twist significantly from planarity.

It should be emphasized that the L chain residues and the atoms involved in the cross-molecule hydrogen bonding in B7-15A2 are the same as those responsible for the ribbons in the crystals of Mcg dimers [51]. Moreover, the critical H chain residues of B7-15A2 are located in comparable positions in the C_H1 sequence, counting backward from the interchain Cys residue. Despite major sequence differences between the L and H chains in this region, the side chains do not interfere with the propensities of the 3-3 polypeptide backbone segments to approach each other closely in the crystal lattice.

4. Discussion

4.1. Lack of a concave combining site in B7-15A2

A correlation between CDR3 length and the presence or absence of a binding cavity between V_H and V_L has been previously observed [52,53]. In the B7-15A2 (this article) and Kol IgG1- λ proteins [13], as well as in the Pot IgM- κ cryoglobulin [14], HCDR3 is the dominant feature of the binding site. As the smallest of the three (two residues shorter than in B7-15A2), the Pot HCDR3 collapses to fill the residual space be-

tween V_L and V_H, but does not extend above the plateau-like external surface of the active site [14]. Its presence eliminates the accessibility of many potential contact residues in the other five CDRs lining the V_L-V_H interface of the antibody. As the largest by three residues relative to B7-15A2, the Kol HCDR3 both fills residual interdomain space and protrudes above its neighbors as a convex knob [13]. This loop is less flexible than the others because the outer six residues are linked at their base by a disulfide bond [26]. HCDR3 of B7-15A2 is intermediate in size between the two and the tip of its loop emerges above the average external surface to form the aliphatic ridge discussed previously (Figs. 3 and 5).

The α -carbon tracings of the Kol, Pot and B7-15A2 proteins give a false impression of an entrance to an internal site between LCDR2 and HCDR3. In reality this hole is blocked by the side chain of Tyr L49 in Pot and Kol or its Phe equivalent in B7-15A2. Although designated as a framework residue, L49 has been identified as a contact residue for protein antigens in the D1.3 anti-lysozyme [54,55] and NC41 anti-neuraminidase antibodies [34]. In NC41, HCDR3 is one residue shorter than that of Pot. Only two or three side chains reach the level of the outer rim, thus giving the binding site a crater appearance.

4.2. Structural constraints and shape modifications

There are distinct differences in the Fab elbow bend angles of four human IgG1- λ immunoglobulins, B7-15A2, Kol [13], New [56] and Mcg [47]. The Fabs of B7-15A2 and Kol are more extended and less constrained than those of New and Mcg. Elbow bend angles for the New and Mcg Fab regions are only 135 and 118°, respectively. It is not clear why the New Fab adopts a more flexed structure than Kol and B7-15A2 but in Mcg the underlying principles are well understood. For example, the elbow bend angle in the intact Mcg IgG1 is heavily influenced by the deletion of the 'hinge region' (15 residues) between the C_H1 and C_H2 domains and its two main consequences [48] which are: (1) tensile forces on the first two do-

mains are increased by the downstream shortening of the H chain; and (2) since the deletion includes the H chain half-cystine usually linked to the L chain, there is a compensatory formation of an L–L inter-chain disulfide bond across the molecular (and crystallographic) two-fold axis of the IgG1 molecule. These two constraints serve to lock the L chains in extended conformations and promote extreme flexion at the V_H – C_H1 junction.

If we consider that these constraints are removed in the B7-15A2 and Kol IgG molecules, the L and H chains are now relatively free to form elongated Fabs with elbow bend angles approaching 180°. Moreover, the disulfide bonds linking the L and H chain do not interfere with independent movements of the two Fab arms or appreciably affect segmental flexibility at the V–C junctions.

In its unliganded (native) conformation, the NC6.8 Fab from a murine IgG2b antibody more closely resembles the Fab structure of B7-15A2 than that of Mcg. As in B7-15A2, the NC6.8 L chain is flexed (dihedral angle of 77°) and the H chain is extended (dihedral angle of 97°) [42,57]. When its ligand (*N*-(*p*-cyanophenyl)-*N'*-(diphenylmethyl)-*N''*-(carboxymethyl) guanidine) is bound, the three-dimensional structure of NC6.8 changes dramatically. As a demonstration of these severe changes, crystals of the native protein dissolve immediately if ligand is added to the suspension. Distinctly different crystals of the ligand–protein complex take their place within minutes. These changes are reflected in reciprocal shifts in the dihedral angles as the L chain becomes an extended subunit (angle of 97°) and the H chain adopts a flexed conformation (angle of 75°). Such reciprocal changes are coupled to adjustments in the elbow bend angle, which decreases by 31° from 184 to 153° during the formation of the complex. It is interesting to speculate whether such shifts might occur on complexation of B7-15A2.

4.3. Designing Fab molecules with a tendency to crystallize

Crystal packing mediated through the antiparallel docking of 3-chain layers of the C_L domains

was first described for the Mcg Bence-Jones dimer (trigonal form), crystallized in ammonium sulfate [51]. Similar packing was later noted for the Fab New, which also has a λ -type L chain [56]. In Mcg, docking was bilateral, in the sense that the 3-3 strands of the C_L domain from chain 1 and 2 of the dimer were involved. Each dimer was therefore bound to two other molecules, one on each side, and the process was repeated indefinitely to produce ribbons of antiparallel, symmetry-related molecules. In the structural analysis of the Pot Fv from a human IgM- κ cryoglobulin [14], we found analogous antiparallel stacking of V_L domains promoted by cross-molecule hydrogen bonding between 4-1 β -strands. Because the 4-1 strand of V_H did not participate in such interactions, discrete dimers of antiparallel Fv molecules were formed in the crystal lattice, but not ribbons.

Results summarized above suggest ways to improve the chances for crystallizing Fabs among synthetic human antibodies and their fragments. The primary step is to promote antiparallel stacking of the C_L and C_H1 domains through cross-molecule hydrogen bonding of the C-terminal 3-3 β -strands. For simplicity, the easiest way to start is to graft a λ -type C_L sequence onto V_L and a $\gamma 1$ -type C_H1 sequence onto V_H . Pairs of critical residues in the 3-3 strands can be placed in sequence positions equidistant from the L-H disulfide bond to ensure that they will be in register in the synthetic antibodies.

The λ - $\gamma 1$ combination of C domains seems to be excellent for inter-domain interactions that are conducive to maintaining the 3-3 segments in orientations where they can form hydrogen bonds on each side of the heterodimer. In the B7-15A2 Fab, the pseudo-two-fold relating the two C domains is 170° and the crossing angle is 91.7°, which we assume are both reasonably close to ideal values for this purpose. The corresponding angles for the Mcg dimer are 180° and 94.2°. There are only 80 van der Waals contacts across the C_L – C_H1 interface in B7-15A2. Together with the inter-chain disulfide bond, these interactions are sufficient to maintain the overall structure of the C_L – C_H1 dimer but are not too restrictive to prevent rotational and translational mobility if necessary for crystallization.

Previous studies have clearly shown that the C_L and C_H1 can move either singly or in concert. In the Mcg IgG1, for example, the center of mass of C_L has moved 15 Å laterally relative to that in the Mcg L chain dimer (same protein in a different setting), although the long axes of the C_L domains have remained parallel in the two proteins [47]. Because of the shortening of the H chain in the Mcg IgG1, the C_H1 domain slants sharply downward toward the Fc region and significantly changes the pattern of interactions with C_L . As a further departure from traditional structures, the 3-3 strand of the C_L domain has too many residues for the space available as it courses upward to form an inter-chain disulfide bond with the opposite L chain. Consequently, the 3-3 segment buckles and balloons out from the main body of C_L as an 8-membered loop (residues 207–214). Thus, the critical region for the formation of cross-molecule β -pleated sheets is thoroughly disrupted in crystals of the Mcg IgG1 protein. It obviously is of prime importance to appreciate the plasticity of the C_L – C_H1 system when trying to incorporate crystallizing propensity into the design of synthetic antibodies.

While the V domains do not interfere with bilateral hydrogen bonding of C domains in the Mcg dimer and B7-15A2 Fab, they clearly assume greater roles in the crystallization of other immunoglobulin fragments: e.g. in a heterologous dimer of the Mcg L chain and another human λ chain (Hud). A comparison of the Mcg and Hud V_L sequences shows 36 substitutions (L. Shan, W. R. Gray, and A.B. Edmundson, unpublished results). The C_L sequences of the two components have only four differences, all of which are well removed from the conserved 3-3 strands.

Under the same set of conditions, the Mcg $\times$ Hud hybrid produces different types of crystals than the Mcg dimer [58–60]. Like the Pot Fv, this hybrid crystallizes in a tetragonal space group in which two molecules are paired in the crystal through antiparallel packing (L. W. Guddat and A. B. Edmundson, unpublished results). In the hybrid, the pairing is mediated through the C_L domains by the recurring mechanism of cross-molecule β -pleated sheet formation between 3-3 strands. Again, the same residues participate in

the hydrogen bonding (Thr L204 and Glu L206) but the interactions are limited to the Mcg components.

It is noteworthy that the sequence differences lead to a marked change in the crossing angle of the V domains from 99.6° in the Mcg dimer to 75.8° in the Mcg $\times$ Hud hybrid, while the crossing angles of the C domains remain roughly the same (94.1 vs 92.9°). Apparently, there are a sufficient number of perturbations in this swing of the V domain to rock the Hud C_L domain out of an orientation favorable for intermolecular hydrogen bonding.

This discussion leads to the question of why the role of the B7-15A2 V_L – V_H dimer is so passive in the crystallization process, while the C domains have virtually free reign to dominate the packing. As illustrated in Fig. 6, the V_L – V_H module displays almost sculptured shape complementarity in the stacking of molecules side-by-side, and perhaps this is sufficient for B7-15A2.

Other pertinent results recently became available while we were completing the X-ray analyses of a related pair of Fabs with human κ -type C_L and $\gamma 1$ -type C_H1 domains. These anti-interferon- γ antibodies were prepared as chimeric (human-mouse) and ‘humanized’ IgG versions by Protein Design Labs (Mountain View, CA) and kindly supplied to us. The chimeric molecules consisted of murine V domains and human C domains. Humanized antibodies contained murine-derived CDRs grafted onto human scaffolding that represented about 88% of the total sequence. Despite species differences in their V domain sequences, the chimeric and humanized Fabs crystallized in the same space group (orthorhombic $P2_12_12_1$) with closely similar unit cell dimensions ($\pm 4\%$).

These results add to the evidence for a secondary role of the V domains and this suggestion was confirmed by examination of the actual packing diagrams (Z.-C. Fan and A.B. Edmundson, unpublished results). Although the interactions of the κ -type C_L and the $\gamma 1$ -type C_H1 did not include 3-3 intermolecular β -pleated sheets, their presence helped guarantee that both the chimeric and humanized Fabs would crystallize in PEG 8000. This is also the crystallizing medium used for the B7-15A2 Fab and the Pot Fv. Both the

Mcg dimer and the Mcg $\times$ Hud hybrid crystallize in ammonium sulfate. Therefore, the crystallizing medium seems to be less critical than the protein chemistry of the immunoglobulin fragments.

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