Crystal structure of a glycosylated Fab from an IgM cryoglobulin with properties of a natural proteolytic antibody

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The 2.6 Å (1 Å = 0.1 nm) resolution structure has been determined for the glycosylated Fab (fragment antigen binding) of an IgM (Yvo) obtained from a subject with Waldenström's macroglobulinaemia. Dynamic light scattering was used to estimate the gel point and monitor the formation of an ordered hydroscopic gel of Yvo IgM upon cooling. If a cryoglobulin forms gels in peripheral tissues and organs, the associated swelling and damage to microvasculature can result in considerable morbidity and mortality. The three-dimensional structure of the branched N-linked oligosaccharide associated with the CH1 domain (first constant domain of heavy chain) is reported. The carbohydrate may act to shield part of the lateral surface of the CH1 domain and crowd the junction between the CH1 and CH2 domains, thereby limiting the segmental flexibility of the Fab arms in intact Yvo IgM, especially at low temperatures. Recently, Yvo IgM was shown to have the properties of a naturally occurring proteolytic antibody [Paul, Karle, Planque, Taguchi, Salas, Nishiyama, Handy, Hunter,

Edmundson and Hanson (2004) *J. Biol. Chem.* **279**, 39611–39619; Planque, Bangale, Song, Karle, Taguchi, Poindexter, Bick, Edmundson, Nishiyama and Paul (2004) *J. Biol. Chem.* **279**, 14024–14032]. The Yvo protein displayed the ability to cleave, by a nucleophilic mechanism, the amide bonds of a variety of serine protease substrates and the gp120 coat protein of HIV. An atypical serine, arginine and glutamate motif is located in the middle of the Yvo antigen-binding site and displays an overall geometry that mimics the classical serine, histidine and aspartate catalytic triad of serine proteases. Our present findings indicate that pre-existing or natural antibodies can utilize at least one novel strategy for the cleavage of peptide bonds.

Key words: antibody crystallography, combinatorial peptide chemistry, cryoglobulin, glycoprotein, IgM, natural proteolytic antibody.

INTRODUCTION

Monoclonal gammopathies such as multiple myeloma and Waldenström's macroglobulinaemia are associated with the production of large quantities of monoclonal immunoglobulins, which often have structural defects causing these proteins to aggregate. An extreme example of immunoglobulin aggregation occurs with cryoglobulinaemia, where antibodies form insoluble aggregates (precipitates or gels) upon cooling and return to solution with warming. Presence in the serum of a cryoglobulin is often an indicator of underlying disease like rheumatoid arthritis, systemic lupus erythematosus, chronic viral infections or B-cell malignancies. Cryoglobulins can be monoclonal (type I) or mixtures of monoclonal and polyclonal components (types II and III) [1]. Since the type I cryoglobulins are homogeneous (in comparison with types II and III), they provide excellent model systems for studies of the aggregation process by analytical techniques. Moreover, their homogeneity is favourable for the production of antigen-binding fragments [Fv (variable fragment) and Fab (fragment antigen binding)] suitable for structural analysis by X-ray diffraction methods.

Antibodies of the IgM class represent a major serum isotype that is the first to be expressed in a primary immune response to antigen. Other isotypes like IgG and IgA are only produced after affinity maturation and class-switching. While crystal structures of numerous antibody fragments of affinity-matured IgG and IgA antibodies have been determined, very few have been solved for members of the IgM isotype [2–6]. Progress in crystallographic examinations of complexes of IgM fragments with antigen has been hampered in part by the low intrinsic affinities displayed by IgM relative to those of other soluble antibody classes (IgG, IgA and IgE). Compared with an IgG antibody, which has two antigen-binding sites, a molecular mass of 150 kDa (6.6 S) and 3 % carbohydrate content by mass, a typical IgM pentamer has ten potential binding sites, a molecular mass >950 kDa (19 S) and 12 % of its mass is attributable to complex N- and O-linked carbohydrates. Previously, we developed methods to produce large single crystals of glycosylated Fab derived by enzymatic cleavage of IgM type I cryoglobulins obtained by plasmapheresis of human subjects (Pot and Yvo) with Waldenström's macroglobulinaemia [7].

Although the antigenic target of Yvo IgM is as yet undefined [8], it is well established that many macroglobulins exhibit

Abbreviations used: AMC, 7-amino-4-methylcoumarin; CDR, complementarity-determining region; CH1 domain, first constant domain of heavy chain; CRA, covalently reactive analogue; D_H , hydrodynamic diameter; DLS, dynamic light scattering; Fab, fragment antigen binding; FR, framework region; Fv, variable fragment; HCDR, heavy CDR; LCDR, light CDR; PDI, polydispersity index; rmsd, root mean square deviation; VH domain, variable domain of heavy chain; VL domain, variable domain of light chain.

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Atomic co-ordinates and structure factors have been deposited in the RCSB (Research Collaborative for Structural Bioinformatics) Protein Data Bank under the accession code 2AGJ.

specificities common to natural antibodies and autoantibodies [9,10]. Recently, Yvo IgM was shown to have the properties of a naturally occurring proteolytic antibody [11,12]. Standard protease substrates cleaved by Yvo IgM include Val-Leu-Lys-AMC (where AMC is the fluorescent reporter molecule 7-amino-4-methylcoumarin) and Glu-Ala-Arg-AMC at rates of 2.6 ± 0.2 and $7.4 \pm 0.3 \mu\text{M} \cdot \text{h}^{-1} \cdot (\mu\text{M IgM})^{-1}$ respectively. Protease activity of Yvo IgM could be inhibited by both a CRA (covalently reactive analogue) of the substrate and standard serine protease inhibitors like di-isopropyl fluorophosphate. The catalytic activity was associated with the Fab portion and thought to be localized in the antigen-binding site [12].

In a recent study of HIV-free humans and mice, Paul et al. [11] identified natural IgM antibodies capable of selective cleavage of the HIV coat protein gp120. Interestingly, of the 12 human monoclonal IgMs tested, Yvo IgM was the most efficient at cleaving gp120. Most of the hydrolysis occurred at the peptide bond between residues Lys⁴³² and Ala⁴³³ of gp120, but minor cleavages at two N-terminal sites were also detected. As in assays with model substrates of serine proteases, the Yvo Fab displayed a dose-dependent cleavage of gp120. A CRA derivative of a gp120 peptide selectively formed a covalent adduct with the κ light chain, indicating a nucleophilic mechanism for the catalytic activity of Yvo IgM.

We report in the present study the crystal structure of a glycosylated Fab from the Yvo IgM cryoglobulin, which reversibly forms a hydroscopic gel upon cooling. A branched N-linked oligosaccharide was found on a loop at the distal end of the CH1 domain (first constant domain of heavy chain) and its core structure was defined. An unusual combination of serine, arginine and glutamate side chains was encountered in the middle of the Yvo antigen-binding site. The spatial arrangement of these side chains resembled the geometry associated with the serine, histidine and aspartate charge relay system in a typical serine protease.

EXPERIMENTAL

Purification of the Yvo IgM cryoglobulin

The Yvo IgM (κ) is a type I monoclonal cryoglobulin, obtained from a human subject undergoing periodic plasmapheresis for Waldenström's macroglobulinaemia. Purification of Yvo IgM involved repetitive cycles of cooling plasma samples from 37 to 4 °C, during which the Yvo IgM forms a semi-solid translucent gel. Following centrifugation, the supernate was removed and replaced with an equal volume of PBS before rewarming to 37 °C. This procedure was repeated at least five times before precipitating the Yvo IgM at room temperature (21 °C) by the addition of solid ammonium sulphate to achieve 45 % saturation. Yvo IgM samples prepared by this method were > 95 % free from contaminating plasma components, as assessed by SDS/PAGE and gel filtration.

DLS (dynamic light scattering) measurements of cold-induced association of Yvo IgM

DLS measurements were performed using a Zetasizer nano ZS (Malvern Instruments), fitted with a 4 mW He-Ne 633 nm laser. Photon-correlated light scattering data were obtained in triplicate using a single detector located at an angle of 173° with respect to the laser beam. The DLS data were collected and analysed with Malvern Instruments DTS (dispersion technology software), package version 3.30. DLS measurements were performed on 2 mg/ml solutions of purified Yvo IgM in PBS across a temperature range of 25–3 °C. The z -average hydrodynamic diameters (D_H), size distributions and PDIs (polydispersity indices)

were estimated from the correlation function after fitting by the Cumulants method implemented in the DTS package.

Combinatorial synthesis and testing of tripeptides for binding to Yvo IgM

Peptides were synthesized as 8×12 arrays on to solid supports (rods) and were tethered by β -alanine spacers following well-established methods [13–15]. Detailed methods for synthesis and ELISA used for Yvo IgM are essentially identical with those previously employed for testing the Mez IgM cryoglobulin [16]. Tripeptide libraries contained lysine at position 1, 2 or 3, and the remaining two positions were filled by combinations of 19 commonly occurring amino acids; arginine was excluded. The latter was replaced by a mixture of all 20 common amino acids (designated as '&'). The Yvo IgM was prepared at a concentration of 0.5 $\mu\text{g/ml}$ in PBS, containing 2 % (w/v) BSA, and ELISA-based assays were performed as previously described [16]. Significant peptide binding values (peaks) were defined as those greater than 3 S.D. above the mean for the lowest 25 % of the values in the assay. To compare independent experiments, the values for the significant peaks were converted into relative binding values (i.e. 0–100), with 100 representing the peptide giving the highest ELISA absorbance reading.

Preparation and crystallization of the Fab of Yvo IgM

Procedures used for the preparation and crystallization of the Fabs from human IgM cryoglobulins Pot and Yvo have been described elsewhere [7]. For production of Fab used in the present study, the Yvo IgM (10 mg/ml) was hydrolysed with trypsin (0.2 mg/ml) in 0.1 M Tris/HCl (pH 8.0) containing 0.01 M CaCl_2 at 56 °C for 4 h. Following the digestion of the Yvo IgM, the $\text{Fc}_{5\mu}$ (pentameric Fc of IgM) fragments precipitated and could be isolated by centrifugation for 20 min at 5856 g (Sorvall). The Yvo Fab was purified by gel filtration (S200; Amersham Biosciences) followed by ion exchange (Mono Q; Amersham Biosciences). Removal of residual sialic acid from the N-linked glycan moiety on the CH1 domain was achieved by treatment of the Yvo Fab with 0.1 unit of agarose-bound neuraminidase (Sigma) per mg of the protein in 50 mM sodium acetate (pH 5.0) at 37 °C for 16 h.

For crystallization, the Fab was concentrated to 26.0 mg/ml in 0.05 % sodium azide in tissue culture-grade water (Sigma). Crystals of Yvo Fab were prepared at 14 °C by vapour diffusion in 8 μl sitting droplets composed of 4 μl each of Fab and reservoir solutions. The 1 ml reservoir contained 85 mM NaCl, 11 % (w/v) poly(ethylene glycol) (15–20 kDa) and 0.1 M Mes buffer (pH 6.5). Under these conditions, single rod-shaped crystals of Yvo Fab typically appeared within 5 days and grew to sizes suitable for X-ray diffraction within 2 weeks.

Collection of X-ray diffraction data

A large (1.5 mm $\times$ 0.8 mm $\times$ 0.3 mm) crystal of Yvo Fab was mounted on a quartz capillary. X-ray diffraction data were collected at 293 K with a Rigaku RU-H3R rotating anode generator coupled with a MAR345 image plate detector. The X-ray beam was collimated to a 0.3 mm diameter with Osmic Max-Flux con-focal mirrors. Data were collected at a crystal-to-detector distance of 200 mm around a single ϕ -axis with 1.0° oscillation per 600 s exposure. Initially, the crystal diffracted X-rays to d -spacings of 2.1 Å (1 Å = 0.1 nm). However, because of significant radiation/thermal damage, it was necessary to move the targeted region of the crystal in the X-ray beam a total of four times during the collection period to obtain a final set of data to 2.6 Å resolution. These data (Table 1) were processed using the DENZO and SCALEPACK programs within the HKL suite, version 1.96.6

Table 1 Data collection and crystallographic refinement statistics

Values in parentheses refer to the highest resolution shell, 2.69–2.60 Å, in the data. Refinement and stereochemical statistics were compiled using the CNS program suite, version 1.0 [19] or PROCHECK, version 3.3 [20].

Parameter	Value
Data collection	
Space group	I222
Cell dimensions (Å)	$a = 82.43$, $b = 103.65$, $c = 138.72$
Resolution range (Å)	99.0–2.60 (2.69–2.6)
Number of unique reflections	18 260 (1742)
Percentage data completeness	98.0 (95.7)
Average multiplicity	8.5 (4.0)
R_{sym}	0.102 (0.854)
Mean $I/\sigma(I)$	32.5 (2.0)
Crystallographic refinement	
R_{work}	0.219
R_{free}	0.279
Average B -factor from Wilson plot (Å ²)	52.8
Estimated coordinate error from Luzzati plot (Å)	0.34
rmsd from ideal values	
Bond lengths (Å)	0.01
Bond angles (°)	1.8
Dihedral angles (°)	27.5
Improper angles (°)	1.2
Ramachandran plot values (%)	
Most favoured regions	75.7
Additional allowed regions	19.3
Generously allowed regions	4.0
Disallowed regions	1.1

(HKL Research, Charlottesville, VA, U.S.A.) [17]. An unusually high R_{sym} (R -factor for symmetry-related intensities) for the 2.69–2.60 Å resolution shell can be attributed to the merging of partially overlapping wedges of X-ray diffraction data collected from different portions of the same crystal. However, the final dataset was highly redundant and the inclusion of intensities to a resolution of 2.6 Å were supported by obvious improvements in the electron density maps.

Structure determination and crystallographic refinement

Neuraminidase-treated Yvo Fab crystallized in the orthorhombic $I222$ space group with unit cell dimensions of $a = 82.43$ Å, $b = 103.65$ Å and $c = 138.72$ Å; $\alpha = \beta = \gamma = 90^\circ$. The Yvo Fab structure was solved using co-ordinates from the Pot IgM Fv ([2]; PDB code 1IGM) and the CL-CH1 domains (where CL is constant domain of light chain) of the Kau Fab ([4]; PDB code 1DN0) as search models for the molecular replacement routine using the AMoRe program [18]. The asymmetric unit consists of a single Yvo Fab molecule. Amino acid residues of the search models were replaced with those of the Yvo Fab prior to crystallographic refinement, which involved repeated cycles of conjugate-gradient energy minimization, simulated annealing and temperature (B) factor refinement within the CNS program suite, version 1.0 [19]. Fitting of the model to electron density maps was accomplished with the TURBO-FRODO program, version 5.5 (BioGraphics, Marseilles, France). After the R_{work} (crystallographic R -factor) and R_{free} (cross-validated crystallographic R -factor) decreased to 0.226 and 0.282 respectively, ordered solvent molecules were built into positive peaks (2.5σ or greater) of $F_o - F_c$ electron density maps. After refinement of individual B factors, the final values for R_{work} and R_{free} converged to 0.219 and 0.279 respectively. A bulk solvent correction and anisotropic overall B factor refinements were applied throughout the structure refinement. The PROCHECK program, version 3.3, was used for structure validation [20]. Rel-

Figure 1 Assessment of cold-induced gelation of Yvo IgM by DLS

(a) Variation of the z -average hydrodynamic diameter (D_H ; solid line) and PDI (dashed line) of a 2 mg/ml Yvo IgM sample cooled from 25 to 3 °C. Mean values ($n = 3$) and S.D. are shown. (b) Intensity distributions obtained by nonlinear least-squares analysis of the correlation functions of Yvo IgM samples maintained at different temperatures.

evant crystallographic refinement values are presented in Table 1. Figures were prepared using the TURBO-FRODO and MOLMOL programs [21].

RESULTS

Hydrodynamic properties of Yvo IgM during cooling indicate the formation of a semi-ordered gel

Previous studies of IgM cryoglobulins utilized sedimentation equilibrium analysis to monitor intermolecular interactions and to identify interaction sites on both Fab $_{\mu}$ and Fc $_{S\mu}$ [22]. Other investigations revealed that individual cryoglobulin samples are influenced by a variety of factors including pH, ionic strength and bivalent metal ions like calcium [23,24]. Thus cryoglobulins exhibit a diverse range of molecular mechanisms for cold-induced assembly of non-covalent polymers (precipitates, gels or crystals) with the rate determined by the initial protein concentrations.

Cold-induced gel formation of Yvo IgM was monitored by DLS (Figure 1). The latter has the decided advantage over analytical ultracentrifugation in not affecting the initial protein concentration. Equilibrium measurements of Yvo IgM at 25 °C indicate a z -average D_H of 42.9 ± 0.09 nm and a PDI of 0.31 ± 0.009 . As the temperature of the IgM samples was lowered from 25 to 3 °C, the z -average D_H increased to 99.8 ± 2.7 nm and the PDI to 0.50 ± 0.005 (see Figure 1a). Upon gelation, the system exhibits a marked decrease in translational mobility, thereby making it possible to estimate a gel point from the intercept of the lines of best fit through the two nearly linear regions of the curve [25]. Using this approach, we estimated a gel point of 10.5 °C for a 2 mg/ml sample of Yvo IgM.

Examination of intensity histograms (Figure 1b) revealed a probable three-stage transition of Yvo IgM samples from: (i) single IgM molecules dispersed evenly in solution; to (ii) clustered IgM molecules forming nuclei in solution; and (iii) a mixture of clustered IgM nuclei and a hydroscopic gel. At 25 °C the intensity

histogram suggests that >99 % of the IgM molecules are freely moving in solution, with residual intensities in a peak around 665 nm, attributable to a small amount (1 % by mass) of aggregated protein. Just above the gel point at 11 °C, a monomodal particle population with a *z*-average D_H of 59.2 ± 0.7 nm and a PDI of 0.34 ± 0.04 indicates that Yvo IgM molecules are interacting to form uniform clusters in perhaps the key nucleation step of gelation. Finally, at temperatures below the gel point (e.g. 5 and 3 °C), Yvo IgM appears to separate into two phases: clustered nuclei (solution phase) and a hydroscopic gel (solid phase).

Amino acid sequences of the Yvo variable domains

A complete sequence of the Yvo VL domain (variable domain of light chain) was obtained by two of us (C. R. Moomaw and C. A. Slaughter, unpublished work). The κ -type light chain has an unblocked N-terminus, which permitted the determination of the first 30 residues by a repetitive Edman degradation method with an automated sequencer (Applied Biosystems Model 470 sequencer and model 120A analyser). Soluble peptides representing the remainder of the VL domain were released by the action of trypsin and fractionated by gel filtration and ion-exchange chromatography. The tryptic peptides were sequenced by a combination of mass spectroscopy and Edman degradation.

Conventional methods were not sufficiently powerful to determine the sequence of the VH domain (variable domain of heavy chain), which failed to yield soluble peptides by typical enzymatic and chemical degradation procedures. Protein sequencing of the VH-derived peptides required exhaustive reduction and carboxymethylation of the Fab, followed by boiling of the insoluble peptide fraction in SDS. Fragments were further separated by SDS/PAGE followed by in-gel digestions and passive differential transfer on to PVDF sequencing membranes. Using this approach, we successfully obtained a fully overlapping sequence for the Yvo VH domain [8].

Figure 2 shows the considerable differences in the amino acid sequences of the Yvo VL and VH domains when compared with the same regions of two other IgM cryoglobulins (Pot and Mez) previously subjected to crystallographic analysis by our group [2,5]. The Yvo κ light chain appears to be the product of the A27 (89 % amino acid identity) $V\kappa 3$ and the $J\kappa 1$ (92 %) gene segments. The heavy chain rearrangement involves the VH2-5 (85 %) gene, the D6-19 (60 %) minigene and the JH4 (87 %) gene segment. Sequence differences among Yvo, Pot and Mez VL and VH domains are primarily attributable to the encoding of the last two by $V\kappa 1$ and VH3 family genes. Of the three cryoglobulins, Yvo contains the shortest HCDR3 (heavy complementary-determining region 3) of only ten residues, compared with the 12- and 14-residue HCDR3 loops of the Pot and Mez proteins respectively (Figure 2). Amino acid sequences for the three HCDR3 loops are comprehensively dissimilar, indicative of distinct preferences for ligand binding by the Yvo, Pot and Mez IgMs.

General features of the crystal structure of the glycosylated Yvo Fab

The Yvo Fab crystallized in the monoclinic $C2$ space group, as well as in two orthorhombic $P2_12_12_1$ and $I222$ space groups. Monoclinic and $P2_12_12_1$ crystals diffracted to *d*-spacings of 4 and 3.0 Å resolution respectively. Although the $I222$ crystals initially diffracted X-rays to at least 2.1 Å resolution at room temperature, they proved to be sensitive to radiation/thermal damage and to exposure to the commonly used cryoprotectants. To compensate for the technical difficulties, the present structure was derived from a 2.6 Å resolution dataset collected at 293 K from a large $I222$ crystal (Table 1). The crystallographic asymmetric unit was a single Fab molecule, the three-dimensional structure of which was

Figure 2 Amino acid sequences of the variable domains of human IgM cryoglobulins

Alignments are: (a) VL domain sequences and (b) VH domain sequences. The numbering scheme and the assignment of FR (framework region) and CDR (boldface) segments are those proposed by Kabat et al. [46]. Amino acid residues identified by lettering are those designated by the Kabat numbering system as insertions. Yvo protein sequences have been aligned with those of the Pot and Mez IgM cryoglobulins, for which crystal structures of Fv portions have previously been determined [2,5]. Amino acid identities are marked by 'star' symbols and gaps by dashes. HFR, heavy framework region; LFR, light framework region.

typical of previously characterized immunoglobulin fragments, including human light chain dimers (Bence Jones proteins) and Fab portions of conventional antibodies [26,27]. The refined atomic co-ordinates submitted to the RCSB Protein Data Bank (accession code 2AGJ) included 215 residues in the light chain, 226 residues in the heavy chain, two GlcNAc residues in the carbohydrate moiety (Figure 3a) and 20 ordered water molecules.

Attempts to transfer Yvo Fab crystals into typical cryoprotectants resulted in rapid cracking or dissolution. However, diffraction data to 2.3 Å were obtained from a small crystal (0.2 mm × 0.2 mm × 0.01 mm) that had been quickly transferred to mother liquor containing 15 % (v/v) 2-methyl-2,4-pentanediol and then flash-cooled to 100 K. The overall completeness of the dataset was 91 % (64 % in the 2.38–2.30 Å range), R_{sym} was 0.049 (0.38), and the average mosaic spread was 0.475°. However, crystallographic

Figure 3 Electron 'cage' density ($F_o - F_c$) contoured at 2.5σ for the N-linked glycan moiety of the Yvo CH1 domain

(a) Only the GlcNAc(β 1-4)GlcNAc segment was observed in the analysis of X-ray diffraction data collected to 2.6 Å resolution at 293 K. (b) In contrast, the branched carbohydrate moiety, Man(α 1-3)[Man(α 1-6)]Man(β 1-4)GlcNAc(β 1-4)GlcNAc, was well defined by modules of electron density at 2.3 Å resolution. The latter map was calculated from X-ray data collected with a small Fab crystal that had been flash-cooled to 100 K.

refinement proved difficult. Currently, the R_{work} and R_{free} are 0.30 and 0.37 respectively, but the calculated electron density maps for the protein correlate very well with the atomic model. Close inspection of the diffraction data revealed that significant numbers of intensities were not acceptable in the low-resolution range of 50–4.6 Å, indicating substantial disorder within the crystal lattice. Despite these problems, data collected at 100 K led to a clearly defined electron density for the branched Man(α 1-3)[Man(α 1-6)]Man(β 1-4)GlcNAc(β 1-4)GlcNAc oligosaccharide attached to Asn^{171H} (Figure 3b). To date, this is the first reported structure of

a branched core region of an oligosaccharide attached to the CH1 domain of an antibody.

Figure 4(a) illustrates the overall three-dimensional structure of the Yvo Fab at 2.6 Å resolution, with the branched N-linked glycan core region presented as space-filling CPK (Corey–Pauling–Koltun) models. When compared with the Pot [2] and Mez [5] IgM cryoglobulins (see below), the Yvo binding site does not display any prominent special features. Its combination of six CDRs are relatively compact and do not protrude outwardly into bulk solvent. A small cavity is found at the interface of the VL and VH domains (Figure 4a).

Amino acid sequences (Figure 2) and conformations of CDRs 1–3 of the light chain as well as CDRs 1 and 2 of the heavy chain are similar to those expected for typical antibodies [28,29]. The 10-membered HCDR3 forms a compact non-protruding loop, with its crest lowered slightly towards the centre of the binding site. Such non-protruding conformations are common for HCDR3s up to 12 residues. Longer HCDR3s tend to loop out beyond the globular boundaries of the VH domain [30]. At the crest of the HCDR3 loop, the side chain of Trp^{98H} partially shields a small cavity with approximate dimensions of 9 Å × 6 Å × 5 Å. This small cavity is suitable for 'end-on' insertion of small ligands or protruding portions (e.g. bulky amino acid side chains) of larger antigens [31,32]. Surfaces around the central binding cavity are provided mainly by side chains from CDRs and are better suited for 'block-end' binding of larger antigens like proteins.

Comparison of the three-dimensional structures of μ - and γ 1-type human CH1 domains

A notable feature of the CH1 domain of the Yvo IgM is the reduced proportion of secondary structure when compared with the CH1 of a human IgG1 (Figures 4b and 4c). Only vestigial β -strands, designated A and G, occur along one surface of the Yvo CH1 domain, and the B and E β -strands are also shortened relative to γ 1-chains. Another obvious difference is the location of the interchain cysteine residue that forms the disulphide bridge with

Figure 4 Overview of the three-dimensional structure of the glycosylated Yvo Fab

(a) Ribbon-style representation with the light chain shown in cyan and the heavy chain shown in magenta. The six CDR segments that define the combining site are indicated as L1–L3 (blue) and H1–H3 (pink). The glycan moiety N-linked to the CH1 domain is depicted as a space-filling model (pale green). Polypeptide chain segments are represented by cylindrical tubes. Strands of β -pleated sheet are shown as directional arrows and helices are denoted by flattened spirals. (b) Glycosylated CH1 domain of Yvo IgM. The branched N-linked carbohydrate is shown as a space-filling model (pale green), together with the asparagine side chain (blue) to which it is attached. (c) CH1 domain of the B7-15A2 Fab (PDB code 1AQK), derived from a human IgG1 antibody with binding specificity for tetanus toxoid [47]. The locations of the cysteine residues involved in light-heavy interchain disulphide bridges are indicated by yellow spheres.

Figure 5 Recognition of lysine-containing tripeptides by Yvo IgM

Grey-scale mapped arrays of absorbance data, in which each cell represents the relative binding strength of Yvo IgM for a single peptide (the background is subtracted for each cell). Data are presented for the following tripeptide libraries: (a) lysine at position 1 (N-terminal); (b) lysine at position 2 (middle); and (c) lysine at position 3 (C-terminal). Positions not occupied by lysine were systematically varied with 19 amino acids, all of which were L-isomers. Arginine was excluded, but was replaced by the mixture ('&') of the naturally occurring residues. Relative binding values (0–100) are indicated by the grey-scale gradient at the far right of the panels.

its partner light chain. This cysteine residue is located in the A strand (N-terminal portion) of μ -type domains, while its location is shifted by more than 70 residues in the linear sequence (C-terminal region) to the G strand of human γ 1-chains. It should be emphasized, however, that the interchain disulphide bonds in IgM and IgG Fabs are situated in close proximity in three-dimensional space.

A common feature of IgM is the presence of an N-glycosylation site in the CH1 domain. In a μ -type CH1 domain, the complex N-linked oligosaccharide is attached to the polypeptide loop that connects the C and D β -strands. This polypeptide segment appears to be quite flexible, as indicated by observations that it is fully or partially disordered in the IgM-derived Fab crystal structures previously determined [3,4,6]. In contrast, the polypeptide loop connecting the C and D β -strands in the Yvo Fab was clearly defined in the electron density maps. The branched N-linked oligosaccharide core region could be partially represented by electron density modules at 293 K and almost fully characterized after collection of X-ray data at 100 K (see above). As illustrated in Figure 4(b), the asparagine-linked GlcNAc(β 1-4)GlcNAc disaccharide segment is packed against the lateral surfaces of the polypeptide loop between β -strands E and F. The presence of the oligosaccharide probably shields this region of the CH1 domain from proteolysis. It almost certainly increases the solubility of the Yvo Fab, which can easily be concentrated to > 100 mg/ml in distilled water. In the isolated Yvo Fab, the remainder of the oligosaccharide extends into bulk solvent, but in the intact IgM is likely to occupy the junction between the CH1 and CH2 domains, thereby influencing their segmental flexibility.

To summarize, the oligosaccharide extension was only observed in crystals maintained at 100 K for data collection. The results may provide insight into possible mechanisms for restricting the flexibility of the CH1 and CH2 domains at their junctions in the intact IgM molecules, particularly at lower temperatures. A currently unaddressed issue is the possible role of the carbohydrate in the formation of the hydroscopic gel of the intact Yvo IgM.

Binding of tripeptides containing lysine

The recent discovery of the serine protease activity in Yvo IgM samples [11,12] led us to re-examine tripeptide binding data for lysine peptides that could also serve as substrates for pancreatic trypsin. These peptides were members of sets originally used for

screening human IgM proteins for binding activity [16] during the development and validation of combinatorial peptide chemistry techniques in H. M. Geysen's laboratory. In the present study, the ELISA data for tripeptide libraries with lysine incorporated into the three possible positions were mapped on a scale of 0–100 in grey-scale on 20×20 arrays (Figure 5). With lysine in the N-terminal position, the ten strongest binding tripeptides were KS&, KQ&, KG&, K&Q, KLG, KSL, KGQ, KN&, KFQ and KAN (absorbance range of 0.878–0.735). The mean and S.D. for the lowest 25 % of the assay was 0.324 ± 0.041 (significant peaks were greater than 0.446). For lysine in the middle position, the order was &K&, SKP, &KM, SKM, QK&, &KQ, SKG, SKI, GKN and PKN (0.764–0.525). The lowest 25 % of the assay was 0.223 ± 0.037 (significant peaks > 0.316). With lysine in the C-terminal position, the ten most potent ligands were T&K, &&K, G&K, SKK, TVK, SQK, TQK, YMK, N&K and ANK (0.696–0.544). The lowest 25 % of the assay was 0.237 ± 0.037 (significant peaks > 0.348). Further studies are required to define the binding specificity and affinities of the various peptide ligands for the Yvo IgM. However, the results presented here do suggest a selectivity of the Yvo-binding site for small lysine-containing peptides. Collectively, the analyses of the binding arrays indicate preferences for alanine, lysine, asparagine, serine, and for threonine in the position adjacent to lysine on its carboxyl side and alanine, lysine, methionine and asparagine preceding lysine (i.e. on its amino side). In most cases, residues with bulky aromatic side chains (e.g. tryptophan and tyrosine) were not favoured when placed next to the lysine residue. Similarly, the Yvo IgM discriminated against acidic aspartate and glutamate residues included in the tripeptide series (Figure 5).

Binding site topographies of three IgM cryoglobulins from human subjects

The Yvo Fab is the third IgM fragment that we selected for X-ray analysis as a guide to structure–function relationships in monoclonal cryoglobulins from patients with Waldenström's macroglobulinaemia. Firstly, a crystal structure was determined for the Fv of Pot IgM at 2.3 Å resolution [2]. Pot IgM binds poorly to peptides, but displays the properties of a polyreactive natural antibody through low-affinity interactions with a list of diverse macromolecules [31,33]. Secondly, we determined the three-dimensional structure of the Mez Fv to 2.6 Å resolution, and were

Figure 6 Depiction of the LCDR3 and HCDR3 loops in three human IgM cryoglobulins

(a) Yvo IgM; (b) Pot IgM (PDB code 1IGM) [2]; and (c) Mez IgM (PDB code 1DQL) [5]. The invariant Phe^{98L} and Trp^{103H} residues are identified by numbers, since they mark the end of the CDR3s and the beginning of FR4 (framework region 4). Hydrogen bonds that occur between LCDR3 (light grey) and HCDR3 (dark grey) residues are shown as dashed lines.

faced with the most prolific peptide binding site ever encountered by our group. Probing the Mez IgM binding site with combinatorial peptide techniques and automated docking procedures netted the probable binding modes for more than 2000 peptide ligands [5,16,34,35]. Thirdly, in the present paper, we show that the Yvo IgM has no extraordinary structural characteristics, but does bind selectively to lysine-containing tripeptides. The latter observation is consistent with the unexpected serine protease activity associated with the Yvo IgM protein [11,12].

The binding properties of the three IgM cryoglobulins are generally commensurate with the conformations and orientations of the HCDR3 loops, which have major effects on the topographies of the combining sites (Figure 6). In Yvo IgM, the orientation of its 10-residue HCDR3 loop relative to LCDR3 (light CDR3) leaves a small cavity at the VL–VH domain interface suitable for the binding of di- or tri-peptide ligands (Figure 6a). Interactions between the LCDR3 and HCDR3 in Yvo IgM are primarily due to ion pairing of Arg^{97L} with Glu^{100aH}. The 12-residue HCDR3 in Pot IgM has collapsed into the VL–VH domain interface, thereby filling all available space and expelling water molecules in the process (Figure 6b). Thus potential contact surfaces formed by side chains of the six CDR loops of Pot IgM are shielded for internal interactions with antigen. The external surface accessible for resulting antigen binding is slightly convex, suggesting that Pot IgM binds macromolecules through low-affinity ‘block-end’ interactions [30]. In marked contrast with the Yvo and Pot proteins, an unusual 14-residue HCDR3 in the Mez IgM protrudes out into the solvent and divides the binding site into two compartments, a large channel between VL and VH and a smaller cavity formed entirely by HCDRs (Figure 6c). Together, these chambers account for the high-avidity binding of a remarkable variety of peptides [16,32,34,35]. As in the Pot protein, multiple interactions (hydrogen bonds and van der Waals) occur between the HCDR3 and LCDR3 loops, but these interactions do not restrict access to ligands in the Mez IgM.

Identification of a putative catalytic triad in the Yvo binding site

Typical naturally occurring serine proteases (e.g. trypsin) contain a catalytic triad of serine, histidine and aspartate, although serine/threonine, basic residue and acidic residue variants have also been documented [36]. Ser–His and His–Asp pairs in positions suitable for charge relay systems are not present in the crystal structure of the Yvo Fab. Since not all human IgMs display

Figure 7 Identification of putative catalytic residues in the Yvo Fab

(a) The serine, arginine and glutamate motif in the binding site of Yvo Fab. (b) The classical serine, histidine and aspartate catalytic triad of bovine trypsin (PDB code 1BJU) [48]. Hydrogen bonds between residues are shown as dashed lines, with distances listed between heavy-atom donor and acceptor atoms. Residues are shaded by atom type, with carbon in light grey, nitrogen in black and oxygen in dark grey.

serine protease activity, subsequent searches of the Yvo variable domains were expanded to identify amino acid substitutions that might qualify as an alternative catalytic triad motif (Figure 2). After examining all combinations of structurally juxtaposed serine or threonine with basic and acidic residues, we identified Ser^{95L}, Arg^{97L} and Glu^{100aH} as a possible trio mediating the proteolytic capabilities of the Yvo IgM. An interesting feature of the putative motif is the participation of residues from both light and heavy chains. As illustrated in Figure 7, the serine hydroxy, arginine

guanidyl and glutamate carboxy groups are positioned in nearly identical arrangements as those in the classical serine, histidine and aspartate catalytic motif, although there are differences in the main chain positions. rmsd (root mean square deviation) values of 2.4 Å were calculated for the three sets of C α atoms, while the rmsd values were only 1.2 Å for the C β atoms. Hydrogen-bonding distances are very similar in the triads of the Yvo IgM and trypsin. The proposed mode is supported by the observation that the side chain of Arg^{97L} lies on the floor of the cavity in a position conducive for direct contacts with any ligand entering the active site of Yvo IgM.

DISCUSSION

Collectively, the three-dimensional structures of antigen-binding fragments (Fab and Fv) of the Yvo, Pot [2] and Mez [5] IgM cryoglobulins have provided glimpses into the structural diversity of the pre-existing or natural immune repertoire of antibodies. The stepwise expansion of HCDRs from 10 (Yvo) to 12 (Pot) to 14 (Mez) residues illustrates how influential these loops are in the overall topographies of the antigen-binding sites. In turn, the alterations in the topographies are associated with surprising changes in binding functions. For example, the Yvo binding site simultaneously presents a relatively large surface area for binding protein antigens, and a small cavity for the binding and enzymatic hydrolysis of short polypeptide segments protruding from proteins like the gp120 molecule of HIV [11]. In the Pot IgM, a slightly convex binding surface with relatively few solvent-exposed side chains is ill equipped to bind small peptides, but promotes low-affinity binding of a wide range of unrelated macromolecules [31,33]. Far from being a large surveillance molecule with limited binding capabilities, the Mez IgM possesses a combination of two cavities lined with an extensive array of side chains facilitating high-avidity binding to at least 2000 synthetic peptides with different sequences [16,34,35]. Even with this limited set of structures, we concluded that binding site diversity of IgM antibodies approaches and sometimes extends beyond the recognition strategies exhibited by typical IgG antibodies. Other publications support this general argument. For instance, the structure of the Fab from an IgM rheumatoid factor revealed an unprecedented interaction, with only the edge of the combining site contacting its Fc antigen [3]. Furthermore, using amino acid sequences and our knowledge of antibody combining site structures, we predicted that diverse binding-site topographies for polyreactive IgM were encoded by germline (or unmutated) immunoglobulin genes [33,37].

In the present paper, we have extended the use of DLS to monitor the formation of hydroscopic gels when Yvo IgM solutions were cooled. The water-retaining properties of cryoglobulins during gelation can be particularly harmful *in vivo*. After exposure to cold temperatures, cryoglobulins precipitate or gel in the capillary beds, causing painful petechiae in peripheral tissues. These local effects are accompanied by damage and swelling in heavily vascularized organs, including the tongue, which can become sufficiently engorged to interfere with breathing and swallowing.

At a concentration of 2 mg/ml, the gel point of Yvo IgM was found to be 10.5°C, a temperature routinely observed in peripheral tissues in cold climates. Formation of the gel follows an ordered process from individual IgM molecules to clustered aggregates or nuclei, which subsequently polymerize to form a semi-solid hydroscopic gel. Molecular mechanisms for the ordered formation of these nuclei and gels of Yvo IgM are currently under investigation. However, the structure and the packing of the Yvo Fab molecules in the crystals suggest that the N-linked

oligosaccharide in the CH1 domain may play a prominent role in the gelation process. Because of its proximity to the CH1–CH2 domain junction, the branched oligosaccharide may reduce the segmental flexibility of the Fab relative to the Fc portions in the intact Yvo IgM.

Naturally occurring catalytic antibodies produced by the immune system are often directed against autoantigens. Although considered to be pathogenic in some instances [38–40], catalytic antibodies may also play beneficial roles by producing hydrogen peroxide, which acts as an antimicrobial agent [41,42]. Hydrogen peroxide production occurs regardless of the source or antigenic specificity of the antibody, and therefore should not be considered a function restricted to the natural antibody repertoire. Proteolytic hydrolysis of gp120 is another matter. The Yvo and other (but not all) naturally occurring IgMs from uninfected individuals cleave the HIV coat protein gp120, but at different rates and different sites. All IgG antibodies tested failed to cleave gp120, an observation consistent with the proposal that antiviral catalytic activity is a property of the pre-existing or natural antibody repertoire [11,43].

The Yvo Fab is the first example of a natural IgM antibody fragment for which the three-dimensional structure has been determined and serine protease activity has been demonstrated [11,12]. We have identified the likely catalytic triad to be a combination of serine, arginine and glutamate, which are located in the centre of the Yvo binding site. Corey and Craik [44] have shown that the classical serine, histidine and aspartate catalytic triad of trypsin can be considerably modified without abrogating proteolytic activity. Both the histidine and aspartate residues can be replaced by a variety of amino acids (including histidine to arginine), although the catalytic activity is diminished to between 0.0004 and 0.04 % of the performance of the wild-type. Only serine was found to be essential for cleavage of peptide bonds by trypsin, and the rates of hydrolysis were increased if a basic residue was adjacent to serine. In a second article, Corey et al. [45] showed that it was possible to restore much of the activity of trypsin with a triad altered by an appropriately placed glutamate or aspartate residue. Finally, with respect to Yvo IgM, it is interesting not only that the histidine and aspartate, of the traditional catalytic triad, are replaced with arginine and glutamate, but also that the catalytic site mimics its surrounding antigen-combining site in having its building blocks supplied by two different chains (light and heavy).

We are grateful to Dr Charles S. Craik (Department of Bioinformatics and Biophysics, University of California San Francisco, San Francisco, CA, U.S.A.) for advice and discussions on possible variations in catalytic residues of serine proteases. This work was supported by grant HR00-093 (to P. A. R. and A. B. E.) from the OCAST (Oklahoma Center for Advancement of Science and Technology; Oklahoma City, OK, U.S.A.), and by grant CA72803 (to A. B. E.) from the National Cancer Institute, the U.S. Department of Health and Human Services.

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Received 27 October 2005/13 January 2006; accepted 20 January 2006

Published as BJ Immediate Publication 20 January 2006, doi:10.1042/BJ20051739