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The crystal structure of rabbit IgG-Fc

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Abbreviations used:

IgG, immunoglobulin G; Fc, fragment crystallisable; Fab, antigen binding fragment; rmsd, root mean square deviation; MBP, mannose binding protein; RA, rheumatoid arthritis; RF, rheumatoid factor

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Abstract

We report the structure of the Fc fragment of rabbit IgG at 1.95 angstrom resolution. Rabbit IgG was the molecule for which Porter established the four-chain, Y-shaped structure of the antibody molecule, and crystals of the Fc ("Fragment crystallisable") were first reported almost 50 years ago in this journal (Porter R.R., 1959, Biochem. J., 73, 119-126). This high-resolution analysis, apparently of the same crystal form, reveals several features of IgG-Fc structure that have not previously been described. More of the lower hinge region is visible in this structure than in others, demonstrating not only the acute bend in the IgG molecule that this region can mediate, as seen in receptor complexes, but also that this region has a tendency to adopt a bent structure even in the absence of receptor. As observed in other IgG-Fc structures, the C γ 2 domains display greater mobility/disorder within the crystals than the Cy3 domains; unexpectedly the structure reveals partial cleavage of both Cy2 intra-domain disulphide bonds, while an alternative conformation for one of the cysteine residues in the intact bridge within the more ordered $C\gamma 3$ domains is observed. The N-linked oligosaccharide chains at Asn-297 are well-defined and reveal two alternative conformations for the galactose units on each of $\alpha(1-6)$ linked branches. The presence of this galactose unit is important for stabilising the structure of the entire branched carbohydrate chain, and its absence correlates with severity of autoimmune conditions such as rheumatoid arthritis in both human clinical studies and in a rabbit model of the disease. Rabbit IgG, through this highresolution structure of its Fc region, thus continues to offer new insights into antibody structure.

Introduction

The rabbit (*Oryctolagus cuniculus*) immunoglobulin gamma (IgG) was one of the first molecules of the immune system to be investigated, and the pioneering studies of Porter produced the very first information about the structure of the antibody molecule. Proteolytic cleavage of rabbit IgG by papain into three fragments, one of which crystallised [1] and was termed Fc (Fragment crystallisable), together with two identical fragments, termed Fab (Fragments antigen-binding), led to the first proposal of the four-chain, Y-shaped structure for IgG [2].

Even before the hetero-tetrameric model of two identical heavy and two identical light chains was developed, the first immunoglobulin allotypes were described for the rabbit *IGHG* gene [3]. This observation led Oudin to hypothesize that IgG production was regulated by two independently segregating loci (named a and b). Several years later these two loci were identified, respectively, in the variable region of the heavy chain and the constant region of the kappa light chain genes, confirming the presence of two distinct protein chains in the molecule.

Moreover, amino acid sequence studies on rabbit γ chains were pivotal in clarifying the nature of immunoglobulin domains [4] and the arrangement of disulphide bonds present in the molecule [5]. Further enzymatic digestion and functional characterisation studies of rabbit IgG-Fc provided the first attempts to map the binding sites for complement and cytophilic activity [6-8]. Later, the same molecule was used as a model for the determination of the composition and topology of the carbohydrate chains present on the Fc fragment [9,10], which play a role in stabilising the structure of the Fc and are required for binding to cell surface receptors.

Despite the key role played by this molecule in the history of immunology, no crystal structure is currently available for the Fc fragment of rabbit IgG. We describe here the 1.95Å resolution structure, one of the highest resolution Fc structures yet reported, from a crystal form that appears identical to the crystals pictured almost 50 years ago by Porter [1].

Experimental

Protein purification

Purified polyclonal rabbit IgG (Sigma-Aldrich, UK) was digested with papain according to the manufacturer's instructions (ImmunoPure Fab preparation kit, Pierce Corporation, USA). The Fab portion was removed from the resulting mixture using a protein A column. Fractions containing the Fc fragment and undigested IgG were then loaded onto a Superdex-200 gel filtration column (Amersham Pharmacia Biotech, UK) equilibrated with 0.5 M Tris pH 7.2, 0.25 M NaCl and 0.1% (w/v) sodium azide. The purity of the eluted sample was assessed by non-reducing SDS-PAGE; a single band indicated the presence of intact dimer (data not shown). Fractions containing the purified Fc were pooled and concentrated to 2 mg/ml at 4°C by centrifugation (Amicon, MWCO 10000, Millipore Corporation, USA) and stored at the same temperature until further use.

Crystallisation of the Fc fragment

Crystallisation conditions for the IgG-Fc fragment have previously been reported [8,9], but initial crystallisation trials using these conditions were unsuccessful. However, crystals were obtained in several conditions from a commercial sparse matrix screen (Crystal Screen I, Hampton Research, USA) and promising hits were further optimized using the hanging drop vapour diffusion method. Conditions that yielded crystals suitable for data collection were as follows: 1 ml of 0.1 M sodium

acetate at pH 4.7 and 2 M sodium formate (reservoir) and 1 μ l protein solution with an equal volume of reservoir (drop). The drops were stored at 18°C and plate-like crystals measuring approximately 0.1 mm by 0.1 mm typically appeared after two days. Their morphology was identical to those pictured [1,6] and characterised [11,12] earlier.

Data collection and processing

Crystals were fished using nylon loops, soaked in a cryoprotectant solution (0.1 M sodium acetate, pH 4.7, 2 M sodium formate and 30% glycerol) and flash-cooled in liquid nitrogen. Data were collected at beamline ID29 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The data were processed with MOSFLM [13], SCALA [14] and the CCP4 program suite [15]. The space group and cell parameters were found to be very similar to those observed previously [12]. Calculation of the Matthews coefficient (2.53; 51.1% solvent) indicated the presence of one Fc molecule per asymmetric unit. Data collection and statistics are presented in Table 1. The structure was solved by molecular replacement with MOLREP [15,16] using protein atoms from the human IgG-Fc structure as a search model (PDB code 1H3T [17]). Refinement was performed initially with REFMAC [18] and later with PHENIX [19]. TLS refinement was implemented using 10 TLS groups per chain, as suggested by the TLSMD server [20]. Cycles of refinement were alternated with rounds of manual model building with COOT [21]. Protein atoms were modelled first, followed by carbohydrate, ligands and water molecules. The refined structure was analysed with COOT, PROCHECK [22] and MolProbity [23]. Secondary structure was assigned with the KSDSSP algorithm implemented in Chimera [24]. Omit maps were prepared in CNS [25]. Figures were prepared with PyMOL (www.pymol.sourceforge.net). Refinement statistics are presented in Table 2.

Atomic coordinates and structure factors have been deposited in the PDB database with the accession code 2VUO.

Results and discussion

Overall structure

The 1.95Å resolution structure of rabbit IgG-Fc displays the typical horseshoe-like arrangement of the C γ 2 and C γ 3 domains in the two polypeptide chains (Figure 1). Superimposition with the highest resolution human IgG-Fc structure (PDB code 1L6X; 73% sequence identity with rabbit IgG-Fc), revealed RMSD values of 0.81 and 0.76 Å for the two chains (performed with LSQMAN software [26]). Superimpositions of the individual domains (RMSD 0.37 and 0.47 Å for C γ 3; 0.80 and 0.85 Å for C γ 2) indicated that most of the differences between the rabbit and human structure occur in the C γ 2 domains.

In the rabbit IgC-Fc structure, interpretable density was present for residues Pro-230 to Ser-444 in chain A, and residues Pro-231 to Ser-444 in chain B; the three C-terminal residues were disordered. At the N-termini, although no density was observed in either chain for Cys-229, the residue involved in the inter-chain disulphide bond, much more of the "lower hinge" region is visible here than in other Fc structures (usually defined from Pro-238), except those complexed with receptors (Fc γ R) that bind to this part of the Fc molecule. However, Pro-230 and Pro-231 (in chains A and B respectively) are only 7.8Å apart (distance between closest atoms), consistent with an intact interchain disulphide bridge between residues Cys-229. In Figure 1, this lower hinge region of the polypeptide chain points towards the viewer, and demonstrates the flexibility and acute bend in the IgG molecule that it can mediate. It is striking that the conformation of this region in rabbit Fc is very similar to that observed in Fc-receptor complexes (PDB codes 1E4K, 1T89, 1T83) [27,28] and

in intact immunoglobulins (1HZH, 1IGT) [29, 30]. In the receptor complexes, a bend in this lower hinge region is demanded by the presence of the receptor. In the rabbit Fc structure, a single hydrogen bond between Glu-233 (chain B) and Arg-443 in the C γ 3 domain of a symmetry-related molecule may assist in stabilising this part of the structure, but the only other crystal packing contacts with any lower hinge region residues on either chain are a total of five van der Waals interactions (Glu-233 on both chains and Leu-235 on chain A; cut-off < 3.5Å). Thus although the presence of a symmetry-related molecule in this crystal form may restrict the conformational freedom of the lower hinge compared with other Fc crystal structures, it appears that this part of the hinge has a natural tendency to adopt this "bent" conformation even in the absence of bound receptor.

The C γ 2 domains are more mobile (or disordered) than the C γ 3 dimer, as reflected by their higher overall B factors (average values 27.8 and 21.2 Å² respectively, Table 2). This feature has been seen in almost all other Fc structures, from the first reported human IgG-Fc [31] to the recent high-resolution structure 1L6X (average values 23.8 and 18.7 Å² respectively). No significant difference in the B values was observed between the two chains of rabbit IgG-Fc however, implying that the values for C γ 2 and C γ 3 reflect an intrinsic difference between the two domains, and are not the result of different crystal packing environments.

The conformation of the main chain for residues 283-296, adjacent to residue Asn-297 to which carbohydrate is covalently attached, has been found to adopt one of two different conformations in other IgG-Fc structures; these have been termed the 'classical' and the 'complex' conformation [32]. In rabbit IgG-Fc, the structure conforms to the 'complex' conformation observed in the recently published high-resolution human IgG-Fc structures [17,32].

Intra-domain disulphide bonds

Each domain contains a conserved intra-chain disulphide bond, bridging the two β -sheets of the immunoglobulin fold. In both C γ 2 domains, the electron density indicates the presence of both an intact covalent bond and a broken state for the Cys-261 to Cys-321 bridge (Figure 2A). The differences in conformation between the two cysteinyl residues derives principally from a rotation around the χ_1 (N-C α -C β -C γ) torsion angle, and are almost certainly the result of radiation damage during data collection. In the C γ 3 domains there is no evidence of intra-domain disulphide bond cleavage, but Cys-425 in chain A (Figure 2B) displays two alternative conformations, indicating that some degree of flexibility is present even in the core of this immunoglobulin domain. The partial cleavage of both bonds within the C γ 2 domains is clearly consistent with the higher B values observed for this domain, as discussed above.

Allelic variation

Allotypes of the *IGHG* gene, which codes for the only IgG heavy chain subclass in the rabbit, have been described in the constant region of the γ heavy chain. They correlate with single amino-acid substitutions at positions 228 and 309 in the hinge and C γ 2 domain respectively [33]. Each locus presents two serologically defined alleles: *d11* and *d12* at position 228 and *e14* and *e15* at position 309. The two allelic variants at position 309, which is defined in the crystal structure, correspond to an Ala/Thr substitution. Given the nature of the rabbit IgG sample, no information was available regarding the sequence or the allelic proportions present in the crystallised protein. As the allelic variant with alanine is the more widespread [34], this residue (corresponding to the *e15* allotype) was refined at position 309. No positive density in the F₀-F_c map (even at a contour level of ~2.0 σ) was observed, suggesting that the allele with Ala was the one exclusively present in the crystal. The presence of an Ala residue at position 309 was later confirmed by mass spectrometry analysis (data not shown). An allotypic variant (nG4m; Leu/Val) is also found at position 309 in human IgG4 [35]. This amino acid is located at the $C\gamma 2/C\gamma 3$ interface, in the binding site for the neonatal Fc receptor [36]. While the two variants characterised in human Fc both code for hydrophobic residues, the *e14* allotype in rabbit Fc codes for a polar residue, which may affect the binding of the neonatal receptor, although there are no reported functional studies that test this hypothesis. Incidentally, we observed positive density on both chains at Ala-396 indicative of a threonine or valine residue, although no variability has been noted previously at this position.

Glycosylation

Rabbit IgG-Fc possesses a conserved glycosylation site at Asn-297 in each C γ 2 domain, at which a complex biantennary type oligosaccharide is covalently attached. The carbohydrate covers a hydrophobic patch on the C γ 2 domain surface and extends into the cavity between the two C γ 2 domains (Figure 3). Its presence stabilises and maintains the structure of the protein, as proposed initially from studies of the human IgG-Fc fragment [31] and later confirmed by microcalorimetry experiments [37,38] and structural studies of sequentially deglycosylated Fc glycoforms [17]. Moreover, the conserved oligosaccharide plays a crucial role in IgG effector functions: it is required for optimal Fc γ receptor binding, and mediates interactions with serum lectins such as mannose binding protein (MBP) that leads to complement activation [39,40].

While the core of the oligosaccharide is conserved, heterogeneity is observed in the fucosylation (found in ~30-40% of rabbit IgG-Fc molecules), galactosylation (~50-60%) and sialylation of the chain (~25%); an additional bisecting N-acetylglucosamine may also be present (~25-30% of rabbit IgG-Fc molecules) [41,42]. On both chains, carbohydrate units up to and including the galactose at the end of each of the $\alpha(1-6)$ branches (Gal-6) were built into good electron density (Figure 3), whereas on the $\alpha(1-3)$ branches, only Man-7 and GlcNAc-8 (the latter only on chain C, Figure 3) were built. The lack of interpretable electron density for fucose, terminal sialic acid or a bisecting N-acetylglucosamine is consistent with the low fractional compositions reported. The $\alpha(1-3)$ branches extend into the area between the two C γ 2 domains and lie close to each other, but none of the contacts between the two chains described previously in the much lower resolution analysis [9] were seen. While some unliganded hun an Fc structures show inter-chain carbohydrate contacts, others do not [43]; whether or not such contacts occur appears to depend upon domain quaternary structure, which in turn may be determined by crystal packing contacts. The $\alpha(1-6)$ branches lie close to the first two strands (A and B) of the C γ 2 domain, their core regions interacting with residues Phe-241, Phe-243 and Glu-265.

The extent of galactosylation in IgG molecules is highly variable, with a reported 40-50% of the molecules entirely lacking galactose in healthy rabbits [41,42]. We observed density at the end of each $\alpha(1-6)$ branch, clearly indicating the presence of a galactose residue (Gal-6). When refined with an occupancy of 100% (as for all the other carbohydrate units), higher B factors and some negative difference electron density indicated a slightly lower occupancy and/or the presence of alternative conformations. (Refinement with occupancy values set between 40 and 60% revealed positive difference electron density). It was clear however, that the principal conformation adopted by Gal-6 is different in the two chains (Figures 1 and 3). In chain A, the O6 atom of Gal-6 points away from the C γ 2 domain, while in chain B, the O6 atom points towards the C γ 2 surface. These two conformations are related by an approximately 180° rotation of the plane of the hexose ring, but in both, Gal-6 lies in the same cavity, forming (different) hydrogen bonds to the same residues Pro-244, Lys-246 (although density for CE and CZ of Lys-246 on chain A is ill-defined), Glu-258 and Thr-260. The galactose conformation in chain A is identical to that seen in the 1.65Å human IgG-Fc (PDB code 1L6X), but no asymmetry was observed in that structure as the two chains are related by

a crystallographic dyad. There is thus some variability in the nature of the interaction between this carbohydrate unit and the protein surface.

Fc galactosylation and functional implications

The galactose content of human IgG-Fc has been shown to correlate inversely with disease progression in rheumatoid arthritis (RA) [44,45] and other auto-immune diseases [46]. Various mechanisms have been proposed: the absence of galactose may expose hydrophobic surfaces that promote Fc-mediated IgG aggregation, or generate new Fc epitopes that are recognised by rheumatoid factor (RF) autoantibodies [47]. The galactose unit is also known to be a key determinant for stabilising the carbohydrate chain's interaction with the protein domain. Its absence leads to greater mobility of the chains as shown by NMR [48] and crystallographic [49] studies, and this enhanced accessibility permits interaction with lectins such as MBP, leading to complement activation [40]. Similar changes in the galactose content of rabbit IgG have been reported after hyperimmunisation, and enhanced avidity of rabbit IgG RF autoantibodies for IgG-Fc was shown to be due to decreased galactose content [50]. In a rabbit model of RA, a long-term immunisation study that followed RF titre, avidity and IgG-Fc galactose content during disease progression showed the same inverse correlation between RF avidity for IgG-Fc and galactose content [51]. The crystal structure of rabbit IgG-Fc has revealed a conformational variability in the mode of interaction of this key carbohydrate residue and the C γ 2 domain, underlining the tenuous nature of this contact and its role in tipping the balance between a mobile carbohydrate chain and an ordered structure in contact with the C γ 2 domain surface.

Rabbit IgG provided the very first insights into antibody structure, and through the high-resolution crystal structure of its Fc region reported here, it continues to reveal new structural details and enhance our understanding of the antibody molecule.

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Tables

Table 1: Data collection and processing statistics

	2
ID29	
ADSC Q315r	
230	
0.976	
1.95	
$P2_{1}(4)$	
a=58.66, b=71.21, c=69.03	
$\alpha = \gamma = 90.00, \beta = 104.78$	
40194	
2.00-1.95	
99.9 (99.9)	
7.3 (7.2)	
17.2 (3.6)	
0.107 (0.449)	
	ID29ADSC Q315r2300.9761.95 $P2_1$ (4)a=58.66, b=71.21, c=69.03 $\alpha=\gamma=90.00, \beta=104.78$ 401942.00-1.9599.9 (99.9)7.3 (7.2)17.2 (3.6)0.107 (0.449)

Table 2: Refinement statistics

Resolution range (Å)	56.7-1.95
No. of: protein atoms	3479
carbohydrate atoms	186
water molecules	398
formate ions	6
azide ions	2
glycerol molecules	4
Average B factors $(Å^2)$	
protein	24.6
carbohydrate	42.5
water molecules	35.1
formate ions	39.9
azide ions	38.3
glycerol molecules	48.5
protein C γ 2 (chain A)	28.5
protein Cγ2 (chain B)	27.0
protein Cγ3 (chain A)	21.8
protein Cγ3 (chain B)	20.5
$R_{\rm cryst}$ (%)	16.9
$R_{\rm free}$ (%) (5% of reflections)	20.4
rmsd bond lengths (Å)	0.004
rmsd bond angles (°)	0.928
Ramachandran plot (% of total	favoured: 99.5, allowed: 0.5,
residues)	disallowed: 0

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Figure legends

Figure 1 Overall structure of rabbit IgG-Fc

In this stereo representation of the structure, the two polypeptide chains are shown in green and blue, with their oligosaccharide chains (indicated as C and D) in yellow. The position of the N-termini on both chains is indicated by the letter N. The N-terminal, lower hinge region in each chain points directly towards the reader.

Figure 2 Conformations of the intra-domain disulphide bridges

Stereo images of the region surrounding the intra-chain disulphide bridges in C γ 2 (**Figure 2A**) and C γ 3 (**Figure 2B**) of chain A. The electron density is calculated from a composite simulated annealing omit map, contoured at 1 σ , generated omitting 4% of the structure per cycle. Carbon atoms are represented in green, oxygen in red, nitrogen in blue and sulphur atoms in orange. The highly conserved tryptophan residues (277 and 381) adjacent to the disulphide bonds can be seen towards the back of the image. In C γ 2 (**Figure 2A**) the disulphide bond is partially broken, while in C γ 3 (**Figure 2B**), alternative conformations for the intact bridge are observed.

Figure 3 The carbohydrate structure of rabbit IgG-Fc

A) Electron density for the N-linked oligosaccharides at Asn-297 in each polypeptide chain, calculated from a composite simulated annealing omit map (contour level: 1σ), is shown together with a representation of the two C γ 2 domains. B) Schematic representation of the complex biantennary oligosaccharide chain present in rabbit IgG-Fc. The residues modelled in the electron density are highlighted in light grey (GlcNAc-8 is only modelled on chain C).

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