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Structural Analysis of Human IgG-Fc Glycoforms Reveals a Correlation Between Glycosylation and Structural Integrity

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Antibodies may be viewed as adaptor molecules that provide a link between humoral and cellular defence mechanisms. Thus, when antigen-specific IgG antibodies form antigen/antibody immune complexes the effectively aggregated IgG can activate a wide range of effector systems. Multiple effector mechanisms result from cellular activation mediated through a family of IgG-Fc receptors differentially expressed on leucocytes. It is established that glycosylation of IgG-Fc is essential for recognition and activation of these ligands. IgG antibodies predominate in human serum and most therapeutic antibodies are of the IgG class.

The IgG-Fc is a homodimer of N-linked glycopeptide chains comprised of two immunoglobulin domains (Cγ2, Cγ3) that dimerise *via* inter-heavy chain disulphide bridges at the N-terminal region and non-covalent interactions between the C-terminal Cγ3 domains. The overall shape of the IgG-Fc is similar to that of a "horseshoe" with a majority of the internal space filled by the oligosaccharide chains, only attached through asparagine residues 297.

To investigate the influence of individual sugar (monosaccharide) residues of the oligosaccharide on the structure and function of IgG-Fc we have compared the structure of "wild-type" glycosylated IgG1-Fc with that of four glycoforms bearing consecutively truncated oligosaccharides. Removal of terminal *N*-acetylglucosamine as well as mannose sugar residues resulted in the largest conformational changes in both the oligosaccharide and in the polypeptide loop containing the N-glycosylation site. The observed conformational changes in the Cγ2 domain affect the interface between IgG-Fc fragments and FcγRs. Furthermore, we observed that the removal of sugar residues permits the mutual approach of Cγ2 domains resulting in the generation of a "closed" conformation; in contrast to the "open" conformation which was observed for the fully galactosylated IgG-Fc, which may be optimal for FcγR binding. These data provide a structural rationale for the previously observed modulation of effector activities reported for this series of proteins.

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Keywords: Fc-fragment; glycosylation; crystal structure; antibody; effector functions

Introduction

Antibodies may be viewed as adaptor molecules that provide a link between humoral and cellular defence mechanisms. Antigen-specific recognition by antibody results in the formation of immune complexes that may activate multiple effector mechanisms, resulting in the removal and destruction of the complex. The immunoglobulin G (IgG) molecule is comprised of three independent protein moieties connected through a flexible linker

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Abbreviations used: ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; ESI-MS, electrospray ionisation mass spectrometry; ADCC, antibody-dependent cellular cytotoxicity; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry.

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Page 000001

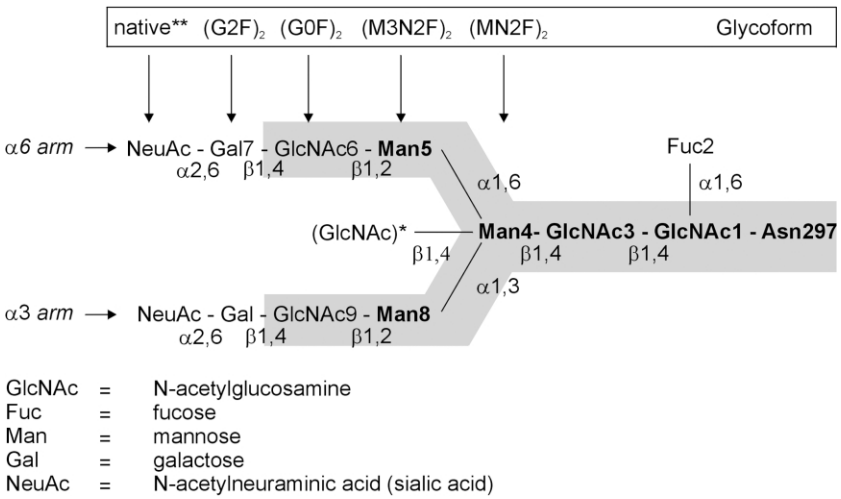

Figure 1. The carbohydrate sequence attached at Asn297 of human IgG1-Fc. Numbers attributes only sugars that have been observed in the electron density. The sugars in bold belong to the oligosaccharide core which is found in all naturally occurring glycoforms, the addition of the other sugar residues is variable. The biantennary mannose residues Man5 and Man8 that are glycosidically linked to Man4 form the α(1–6) and α(1–3) arms, respectively. A bisecting (GlcNAc)* occurs naturally in 5–10% of normal human IgG but not in IgG Cri used here. The arrows point to the termini of respective glycoforms that have been prepared by enzymatic truncation of native IgG-Fc fragments. The native** oligisaccharide is not fully sialylated, but is a mixture of G0F, G1F, G2F and monosialylated one.

or hinge region. Two of these moieties are of identical structure and each expresses an antigen-specific binding site, the Fab regions; the third, or IgG-Fc region, expresses interaction sites for ligands that activate clearance mechanisms. The hinge region allows for segmental flexibility such that each Fab may access and bind its target antigen whilst the Fc remains accessible to effector ligands. The principal ligands through which effector pathways are initiated are cellular Fc receptors and the C1 component of complement. The aggregated forms of IgG-Fc present in immune complexes engage and cross-link the membrane bound Fcγ-Receptors (FcγRs), to initiate an activating or inhibitory signal. Activating signals are mediated through receptors expressing an immunoreceptor tyrosine-based activation motif (ITAM) or inhibitory signals through receptors expressing an immunoreceptor tyrosine-based inhibitory motif (ITIM), within or associated with the cytoplasmic part of the FcγR.[1] FcγRs are constitutively expressed on leucocytes, e.g. B-cells, T-cells, NK-cells, macrophages, eosinophils, neutrophils and mast cells, etc. The consequences of cellular activation by immune complexes depend on the nature of the immune complex formed, the isotype distribution of the specific antibody and the cell type; effector responses include antibody-dependent cellular cytotoxicity (ADCC),[2] secretion of inflammatory mediators,[3] enhanced antigen presentation, regulation of antibody production,[4,5] the oxidative burst[6] and phagocytosis.[7] FcγRs are type I transmembrane proteins and belong to the immunoglobulin superfamily. In humans there are three classes of FcγRs: the high affinity IgG receptor FcγRI ($K_D \approx 10^{-8}$ M) and two low affinity receptors FcγRII and FcγRIII ($K_D \approx 10^{-6}$–10^{-7} M), which are responsible for the clearance of immune complexes.[8] They occur in various isoforms (FcγRIa, -b1, -b2, -c; FcγRIIa1, -2, -b1-3, -c) and there exists a significant polymorphism within the human population (FcγRIIa1-HR, -LR; FcγRIIIb-NA1, -NA2).[9]

Comparison of intact murine and human IgG structures reveals extreme asymmetry and strong interdomain flexibility within the antibody.[10] The homodimeric Fc fragment of IgG has a horseshoe-like structure and is glycosylated at the Asn297 residues, located in the Cγ2 domains. The oligosaccharide is of the complex type and is comprised of a "core" heptasaccharide (Figure 1) that may be extended by the addition of fucose to the primary *N*-acetylglucosamine (GlcNAc) residue and galactose to the arm GlcNAc residues. Galactosylated oligosaccharides may be further extended by the addition of sialic acid. The predominant core structure observed for human IgG is the octasaccharide $FucGlcNAc_2Man_3GlcNAc_2$ which may be extended by the variable addition of galactose and sialic acid residues[11] (Figure 1). The structure of the oligosaccharide moiety reveals branching oligosaccharide arms at the Man4 residue (Figure 1). The α(1–6) arm (Figure 1), branching from Man4, is directed towards the Cγ2–Cγ3 interface region and makes multiple interactions with the surface of the Cγ2 domain, whereas the α(1–3) arm extends into the space between the Cγ2 domains and contacts the α(1–3) arm of the opposing Cγ2 domain (Figure 2(a) and (b)). The sugars of the oligosaccharide trunk and the α(1–6) arm make multiple non-covalent interactions with the protein surface of the Cγ2 domain, predominantly through hydrophobic amino acid residues.[12–14]

Complete N-deglycosylation of IgG1 results in a loss of its capacity to bind FcγRs[15–18] and consequently to a failure in the initiation of the corresponding effector functions.[19] The analysis of the binding affinity of various truncated glycoforms of IgG-Fc demonstrated a progressive decrease in the affinity of binding to soluble recombinant FcγRIIb.[20,21] Although these biochemical data characterise the effects of particular sugar residues,

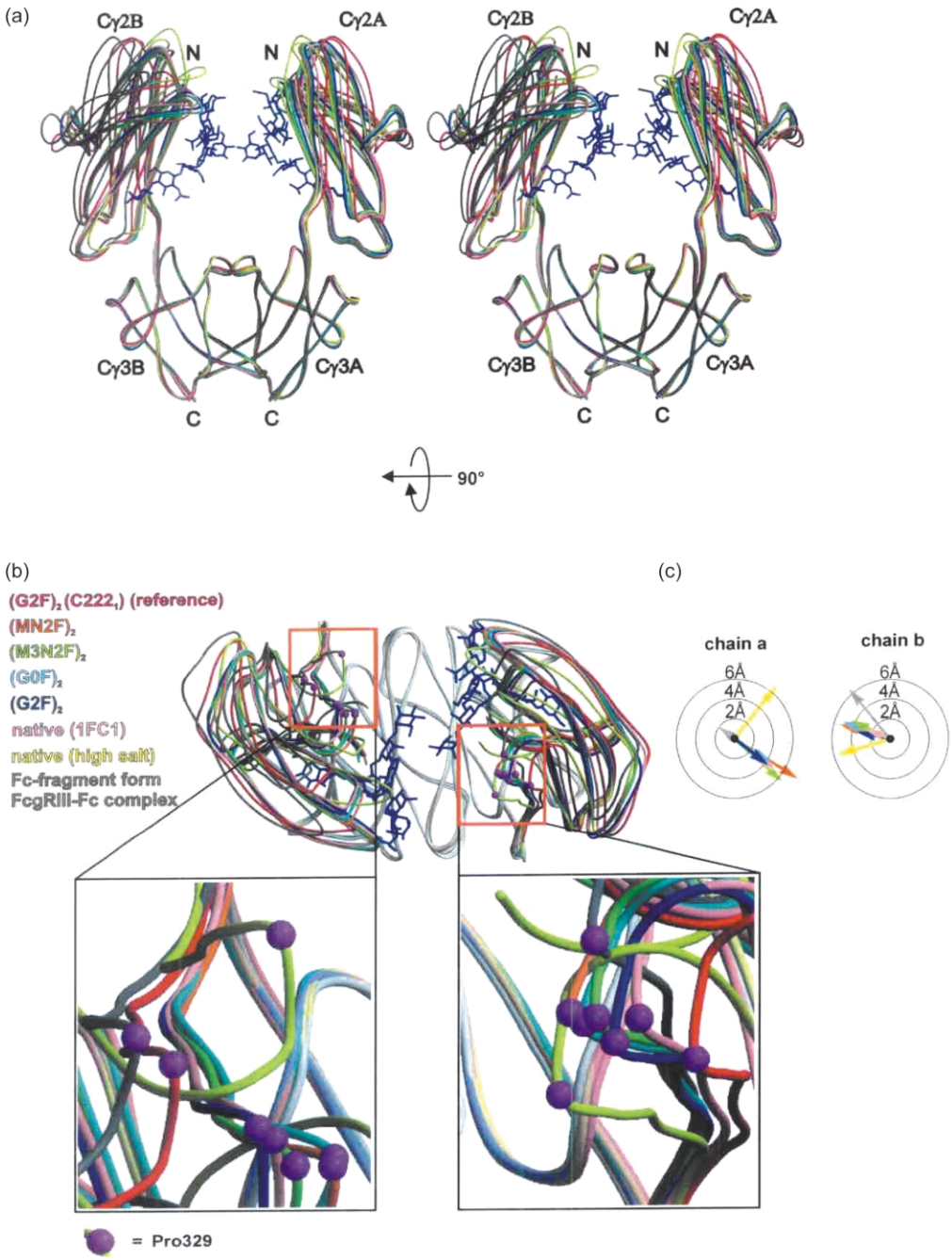

Figure 2. (a) Stereo front view of various Fc-glycoforms and the Fc-fragment in complex with FcγRIII. A superimposition of Cγ3 domains is shown. The colour assignment in (b) applies to (a) and (b). For glycoform $(G2F)_2$ in space group $P2_12_12_1$ the carbohydrate moieties are shown. The positional deviation of Cγ2 domains results from asymmetric tilts of the entire Cγ2 domains around the Cγ2-Cγ3 joint. (b) Top view. The Pro329 residues on the tip of the Cγ2 domains of different IgG-Fc glycoforms are marked as spheres in magenta. (c) The arrows in the vector diagram run from Pro329 of the glycoform $(G2F)_2$ in space group $C222_1$ to Pro329 of all other structures and illustrate the extent of conformational freedom of Cγ2 domains relative to the reference structure.

the precise manner in which the oligosaccharide influences the Fc-fragment structure and function remains unclear.

Given a virtually infinite repertoire of antibody molecules we can anticipate the development of a huge catalogue of antibody therapeutics. Optimal therapeutic efficacy will depend on the specificity and affinity for antigen recognition and the effector activities triggered *in vivo*. Some treatment protocols may attempt to maximise effector function activation whilst for others down regulation may be considered to be of benefit.

Detailed information about the interaction of the IgG-Fc with recombinant soluble FcγRIII was obtained from the crystal structure of the IgG1-Fc-fragment in complex with the FcγRIII.[22] Interestingly only one minor contact between a sugar residue

Table 1. Summary of crystallographic data

Glycoform (Figure 1) PDB-code	Space group	Crystallisation method	Cell constants (cell dimensions in Å)	*R*-factor (%), *R*-free (%)	Resolution (Å)
$(MN2F)_2$	$P2_12_12_1$	Dialysis H_2O	$a = 49.57$ Å, $b = 80.78$Å, $c = 139.65$Å, $\alpha = \beta = \gamma = 90°$	28.0, 30.9	2.4
$(M3N2F)_2$	$P2_12_12_1$	Dialysis H_2O	$a = 49.48$ Å, $b = 79.63$ Å, $c = 143.86$ Å, $\alpha = \beta = \gamma = 90°$	25.3, 28.9	2.4
$(G0F)_2$	$P2_12_12_1$	Dialysis H_2O	$a = 49.69$ Å, $b = 80.20$ Å, $c = 138.96$ Å, $\alpha = \beta = \gamma = 90°$	27.8, 32.9	2.44
$(G2F)_2$	$P2_12_12_1$	Dialysis H_2O	$a = 49.47$ Å, $b = 80.85$ Å, $c = 141.75$ Å, $\alpha = \beta = \gamma = 90°$	24.0, 31.4	3.1
$(G2F)_2$	$C222_1$	Dialysis H_2O	$a = 49.55$ Å, $b = 149.25$ Å, $c = 75.75$ Å, $\alpha = \beta = \gamma = 90°$	27.9, 32.0	2.82
Native high salt	$P6_122$	Vapour diffusion	$a = 152.94$ Å, $b = 152.94$ Å, $c = 116.96$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$	29.2, 35.2	4.1
Native	$P2_12_12_1$	Dialysis H_2O	$a = 80.40$ Å, $b = 146.40$ Å, $c = 50.40$ Å, $\alpha = \beta = \gamma = 90°$	22.0, ND	2.9
Fc-FcγRIII	$P6_522$	Vapour diffusion	$a = 115.32$ Å, $b = 115.32$ Å, $c = 299.10$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$	27.1, 35.7	3.2

PDB code	Mol. P. AU/sol. cont. (%)	No. indep. refl./mult.	Completeness over. (%)/ last shell (%)	R_{merge} overall (%)/ last shell (%)	RMSD bonds (Å)/ RMSD angles (deg.)	Overall *B*-factor (Å^2)
1H3T	1/55	21295/4	98.1/93.4	6/20	0.008/1.5	45
1H3U	1/55	22481/4	97.9/88.9	4.6/30.9	0.007/1.4	50
1H3X	1/55	19885/3.2	93.6/72.8	7.4/41.2	0.008/1.4	51
1H3V	1/54	8183/2	82.8/81.8	13.1/38.4	0.009/1.5	32
1H3W	0.5/77	6718/2	97.5/75.6	8.2/53.8	0.008/1.4	50
1H3Y	4/67	5219/3	80.0/67	18.1/25.5	0.014/2.0	30
1FC1	1/54	10342/2	75/37	7.7/ND	0.007/1.7	30
1E4K	1/67	18596/3.4	94.9/66.8	13.3/60.7	0.016/2.2	95

ND, Not determined value.

and the FcγRIII was observed. Since the activation of FcγRIII is dependent on IgG-Fc glycosylation it is apparent that the oligosaccharide moiety exerts its influence, indirectly, through modulation of IgG-Fc conformation. We have, therefore, investigated the crystal structure of a series of homogeneous, truncated glycoforms of IgG-Fc and interpreted our findings in relation to our previous studies of their influence on Fc receptor recognition and activation.

Results

We determined the crystal structures of four glycoforms of IgG1-Fc generated by the selective activities of glycosidases or glycosyltransferases and compared them with the structure of the native IgG-Fc fragment. Deglycosylated IgG1-Fc was prepared and purified to homogeneity but could not be crystallised under the conditions that were successful for other glycoforms, probably indicative of increased internal disorder of the Cγ2 domains or multiple alternative conformations. Additional screening procedures for new crystal conditions of completely deglycosylated Fc were not successful.

In all solved structures amino acid residues Pro238 to Leu443 could be traced in the electron density of the Fc-fragments. No electron density was observed for the hinge region N-terminal to Pro238, which is disordered in all crystal structures of isolated Fc-fragments.

The extent of sugar trees found in the crystal structures of the Fc glycoforms $(MN2F)_2$ and $(M3N2F)_2$ correspond to the molecular masses

IPR2025-00604
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Page 000004

Table 2. Cγ2–Cγ2 and Man4–Man4 distance in different Fc-glycoforms

Glycoform (space group)	Number of monosaccharides per chain	Pro329 distance Cγ2A–Cγ2B (Å)	Man4 distance Cγ2A–Cγ2B (Å)
$(MN2F)_2$ ($P2_12_12_1$)	4	21.9	13.0
$(M3N2F)_2$ ($P2_12_12_1$)	6	24.2	12.9
$(G0F)_2$ ($P2_12_12_1$)	8	22.6	12.6
$(G2F)_2$ ($P2_12_12_1$)	10	26.6	12.7
$(G2F)_2$ ($C222_1$)	10	33.7	16.7
Native 1FC1	8–11	23.8	12.8
Native high salt	8–11	29.6	7.6
Fc-FcγRIII native	8–11	30.3	16.0

The spacing between Cγ2 domains is specified by the C^{α}-carbon distance between Pro329A and Pro329B. The spacing between the carbohydrate moiety of both chains is specified by the C1-carbon distance of Man4 in chain A and chain B (Man4A, Man4B). The Fc-fragment structure of Deisenhofer (PDB-code: 1FC1) was comprised in the structural comparison.[13]

predicted by electrospray ionisation mass spectrometry (ESI-MS).[21] In the electron density of native IgG-Fc and glycoform $(G2F)_2$ we could trace the carbohydrate tree up to the Gal7 in the α(1–6) arm and up to the GlcNAc9 in the α(1–3) arm (Figure 1). Although the presence of galactose in the α(1–3) arm of glycoform $(G2F)_2$ and of native Fc was confirmed by ESI-MS[21] it yielded no electron density due to flexibility.

IgG-Fc fragment data recorded at 100 K show relatively high *R*-factors (Table 1) that were independent of the space group and consistent with other IgG-Fc structures and the IgG-Fc/FcγRIII complex.[22] The elevated *R*-factors observed may be due to partial disorder introduced into the crystals during the freezing procedure. In contrast data acquisition at room temperature results in lower *R*-factors.[13]

Conformational freedom of Cγ2 domains is influenced by the oligosaccharide

The superposition of the solved IgG-Fc glycoform structures, native IgG-Fc (crystallised at high salt conditions) and IgG-Fc from the complex with FcγRIII reveals that the Cγ3 domains are symmetrically arranged and their conformation is independent of space group, crystallisation conditions and the presence or absence of IgG-Fc receptors. In contrast the Cγ2 domains can "tilt" around the Cγ2–Cγ3 joint, as rigid bodies. In order to illustrate the positional changes of the Cγ2 domains in the different IgG-Fc structures we chose Pro329, which is located in a short rigid loop at the tip of the Fc fragment, as the reference point to reveal maximal positional differences. (Table 2, Figure 2(b)).

We describe the rigid body movement of Cγ2 domains in different IgG-Fc structures relative to a reference structure, namely glycoform $(G2F)_2$ in space group $C222_1$, because here the Cγ2 domains are related by a twofold crystallographic symmetry which makes the structure an ideal tool for the evaluation of the asymmetric tilt of Cγ2 domains in the other IgG-Fc structures. The observed tilt of the Cγ2 domain determines the opening of the horseshoe shaped IgG-Fc fragment (Figure 2(a) and (b)). The dislocation of the Cγ2 domains (represented by the Pro329 dislocation) is depicted in Figure 2(c) in a diagram where the Pro329 position of the symmetric IgG-Fc structure in space group $C222_1$ defines the origin. The individual vectors represent positional Cγ2 domain deviations of the glycoforms analysed with regard to the reference structure $(G2F)_2$ in space group $C222_1$. In the native IgG-Fc fragment, crystallised under high salt conditions, the Cγ2B domain deviates by more than 6 Å with respect to the reference structure, which is due to particular crystal contacts. This reorganisation results in different sugar–sugar contacts between the α(1–3) arms of the oligosaccharide namely GlcNAc9A-Fuc2B, GlcNac9A-Man5B, Man8A-Man5B and GlcNac3A-Man8B, whilst the previously observed contacts between Man8 of both chains is no longer observed.

Analysis of the IgG-Fc glycoform structures crystallised under low salt conditions in space group $P2_12_12_1$ revealed Cγ2–Cγ2 distances comparably small (Figure 2 and Table 2). In the glycoform $(G2F)_2$, with a deca-saccharide structure on each Cγ2 domain, the distance between Cγ2 domains was maximal (26.6 Å), whereas in glycoform $(MN2F)_2$, with a tetrasaccharide structure on each Cγ2 domain, it was minimal (21.9 Å, Table 2). These results show that truncation of the branch chain sugars allows for the mutual approach of Cγ2 domains of the horseshoe-shaped IgG-Fc fragment by 4.7 Å. Intermediate Cγ2–Cγ2 distances were observed for the $(G0F)_2$ and $(M3N2F)_2$ glycoforms, with octasaccharide and hexasaccharide structures attached at each Cγ2 domain, respectively.

In the glycoform $(MN2F)_2$ lacking the branching Man residues no direct contact between the oligosaccharide trunks is observed, whereas in all other IgG-Fc glycoform structures in space group $P2_12_12_1$ Man8 mediates sugar contacts between both Cγ2 domains, as previously shown.[12]

In summary the crystal structures show that the Cγ2–Cγ3 joint permits a high angular freedom to the entire Cγ2 domain while the carbohydrate residues determine a minimum Cγ2 domain distance.

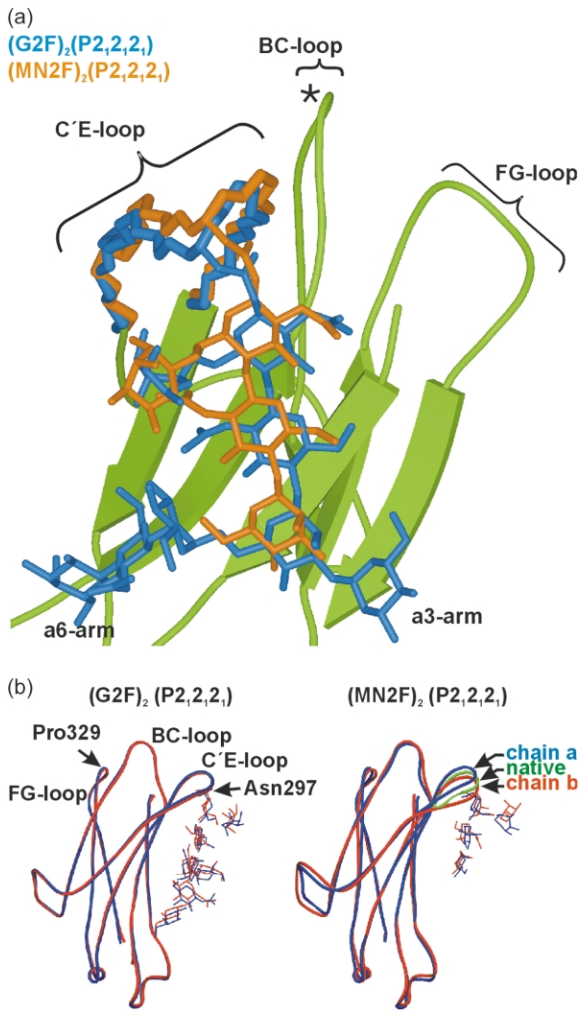

Figure 3. (a) Conformational change of the C′E-loop in the Cγ2A domains upon truncation. Glycoform $(MN2F)_2$ (orange) resembles the conformation of glycoform $(M3N2F)_2$. Glycoform $(G2F)_2$ (blue) resembles the native Fc-structure, all glycoforms carrying oligosaccharides which include at least GlcNAc6/GlcNAc9 and the IgG-Fc fragment in the IgG-Fc–FcγRIII complex. Secondary structure elements of the Cγ2A domains of glycoform $(G2F)_2$ are shown in green. An asterisk marks the position of His268. (b) Superposition of Cγ2A and Cγ2B of $(G2F)_2$ (left) and $(MN2F)_2$ (right). Left: identical C′E-loop conformations are found in both chains of glycoform $(G2F)_2$ ($P2_12_12_1$) carrying ten sugars in the oligosaccharide chain which are similar to the C′E loop conformation of native Fc. Right: in the structure of glycoform $(MN2F)_2$ ($P2_12_12_1$) the C′E-loops shift in opposite directions in both Cγ2 domains compared to the C′E-loop in native Fc (green).

Structural changes within the Cγ2 domains upon truncation of the carbohydrate moieties

A superposition of the Cγ2A and Cγ2B domain of the various glycoforms shows that upon oligosaccharide truncation the only structural changes within the Cγ2 protein occur within the C′E-loops.

The native IgG-Fc fragment, the IgG-Fc fragment in complex with FcγRIII and all glycoforms carrying GlcNAc6/GlcNAc9 or more sugars in their oligosaccharide tree do not show these differences in C′E-loop and oligosaccharide position. In Figure 3(a) these IgG-Fc structures are represented by the structure of $(G2F)_2$. In the glycoforms $(MN2F)_2$ (Figure 3(a)) and $(M3N2F)_2$, lacking GlcNAc6 the C′E-loop conformation and the position of the oligosaccharide differs in Cγ2A with respect to the native C′E-loop structure by 1.5 Å and 1.13 Å, respectively (Figure 3(b)). Similar structural differences are observed for Cγ2B domains. In glycoform $(M3N2F)_2$ the smaller C′E-loop deviation from the native position shows that the branching sugars Man5 and Man8 contribute to the conformational stabilisation of the C′E-loop, in addition to that observed for the GlcNAc residues on the branching arms.

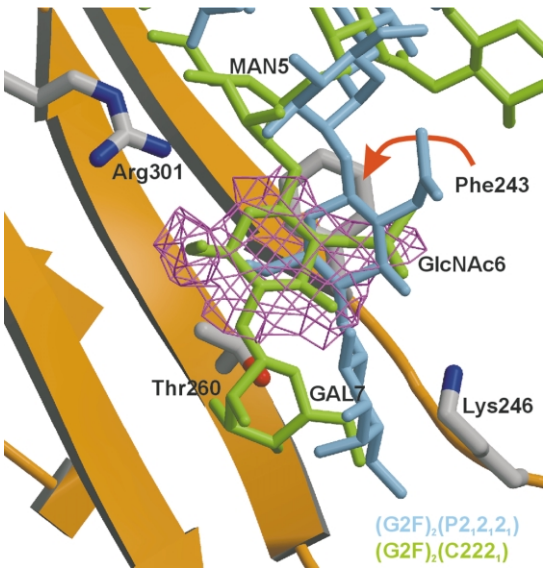

Figure 4. The role of GlcNAc6 in stabilisation of carbohydrate–protein interactions. The structure of the oligosaccharide moiety (chain A) of Fc-glycoform $(G2F)_2$ in space group $C222_1$ (green) and $P2_12_12_1$ (blue) are shown. In contrast to Man5 and Gal7, GlcAc6 maintains a contact to the Cγ2 domain in both structures. For GlcNAc6 of the structure in space group $C222_1$ the $2F_o - F_c$ electron density map is shown contoured at 0.8σ.

The superposition of Cγ2A and Cγ2B domains of a given glycoform with the native IgG-Fc (Figure 3(b)) illustrates that the structural deviations of the C′E-loops are in inverse directions in chain A and chain B. This effect has been observed for the glycoforms $(M3N2F)_2$ and $(MN2F)_2$ in which the C′E loop and the oligosaccharides, that are associated by multiple weak interactions (Supplementary Materials Table 4) to the protein, perform a concerted movement. Although the structure of the oligosaccharide moieties missing α(1–3) and α(1–6) arm sugars is well defined, the core sugars GlcNAc1, GlcNAc3, and Man4 are not sufficient for C′E-loop stabilisation (Figure 3(a)).

Interestingly the protein association of the α(1–6) arm sugar residues Man5 and Gal7 differs between the crystal forms (Figure 4). Thus, in

Table 3. Correlation of carbohydrate chain length and *B*-factors

Glycoform (spacegroup)	Number of monosaccharides per chain	$B_{average}$ chain A		$B_{average}$ chain B		$B_{average}$ carbohy. chain A	$B_{average}$ carbohy. chain B
		Cγ2	Cγ3	Cγ2	Cγ3		
$(MN2F)_2$ ($P2_12_12_1$)	4	38.4	35.6	71.3	32.8	63.3	101.4
$(M3N2F)_2$ ($P2_12_12_1$)	6	46.8	43.4	68.8	42.1	63.3	91.6
$(G0F)_2$ ($P2_12_12_1$)	8	41.6	46.0	72.8	39.2	59.2	90.5
$(G2F)_2$ ($P2_12_12_1$)	10	31.2	22.9	51.8	23.0	48.4	79.4
$(G2F)_2$ ($C222_1$)	10	55.1	44.8	55.1	44.8	94.0	94.0
Native high salt	8–11	49.2	30.5	17.7	20.8	74.2	36.3
Native 1FC1	8–11	24.2	19.1	22.5	18.8	29.0	45.2
Fc-FcγRIII native	8–11	106.0	116.2	68.3	112.1	100.9	77.9

$(G2F)_2$ $C222_1$ both sugars are closely associated with the protein surface, whereas for the same glycoform in a different space group ($P2_12_12_1$) Man5 and Gal7 are 90° rotated in a protein distal conformation (Figure 4). In contrast GlcNAc6 of the α(1–6) arm is always found in contact with Phe243, Thr260 and Lys246 even though its position shifts by about 2 Å on the protein surface (Figure 4). Together with the observation that the C′E-loop conformation changes upon truncation of GlcNAc6 these results indicate that GlcNAc6 is important for the stabilisation of the Cγ2 domain and the contact between oligosaccharide and protein.

Analysis of crystal contacts and *B*-factors

Consistent with the structure of Deisenhofer,[13] higher *B*-factors were observed in one of the two chains (chain B) of all structures of space group $P2_12_12_1$ (Table 3). Different crystal contacts in Cγ2A and Cγ2B domains in three areas influence the extent of thermal motion of both domains which explains the observed difference of *B*-factors in chain A and chain B (Table 3). Although the crystal contacts differ in detail between chain A and chain B they are localised to the same areas in each chain. The first crystal contact is close to the N-glycosylation site Asn297, the second one is at residue Arg255 near the Cγ2–Cγ3 interface and the third one is localised in the loop between Val282 and Thr289. The crystal contact at the loop Val282-Thr289 of chain A and B causes local disorder, resulting in a lack of electron density for His285 in all IgG-Fc structures in space group $P2_12_12_1$ as well as elevated *B*-factors and unfavourable dihedral angles for the residues flanking His285.

In general the oligosaccharides of all solved IgG-Fc structures show a higher level of flexibility than the protein moiety, indicated by elevated *B*-factors (Table 3). Moreover, we observed an inverse correlation between the length of the oligosaccharide chains and the thermal motion of the oligosaccharides and the C′E-loop (data not shown): longer oligosaccharides have lower *B*-factors and shorter oligosaccharides have higher *B*-factors which is due to the net loss of stabilising oligosaccharides/protein interactions following truncation (Table 3).

Discussion

In the recently solved crystal structure of an IgG1-Fc in complex with soluble, recombinant FcγRIII only one unshielded, and therefore unstable, hydrogen bond formed between FcγRIII and the oligosaccharide of chain B of the IgG-Fc fragment was evident. Since there is evidence that FcγRIII and FcRγII interact with IgG-Fc at overlapping, though non-identical, sites it is surprising that no binding was observed between deglycosylated IgG1-Fc and FcγRIIb, using microcalorimetric and BIAcore techniques.[21] If we assume that the IgG-Fc fragment binds to FcγRIIb and FcγRIII in a similar way these results suggest that although direct contacts between oligosaccharide and receptor are not essential for the formation of the complex there is a clear correlation between IgG-Fc glycosylation and the strength of receptor binding.

Our aim in the present study was to find an explanation for this behaviour. Do oligosaccharides stabilise particular conformations of Cγ2 domains? Or do they simply act as spacers, holding both Cγ2 domains apart and favouring an open state of the horseshoe-shaped IgG-Fc fragment facilitating FcγR binding?

Sugars determine the "opening" of the horseshoe-shaped Fc-fragment

The superposition of Cγ3 domains of all solved IgG-Fc glycoform structures (Figure 2(a) and (b)) illustrates that Cγ3 domains are rigid and do not change position between different Fc-structures, whereas the position of Cγ2 domains varies between different IgG-Fc structures. The observed movement is asymmetrical and also found in structures of IgE-Fc, making it a common feature of Fc structures.[23] The observed asymmetric deviations of Cγ2 positions are large in structures of different space groups (for the glycoform $(G2F)_2$ ($C222_1$) 33.7 Å while in space group $(G2F)_2$ ($P2_12_12_1$), 26.6 Å). Therefore, to evaluate the effect of glycosylation on the IgG-Fc opening we compared the

IPR2025-00604
Patent Owner's Exhibit 2023
Page 000007

Cγ2–Cγ2 distances of IgG-Fc glycoform structures of space group $P2_12_12_1$ only (Table 2; Figure 2(c)). We observed that the progressive removal of the sugar residues leads to an approach of the two Cγ2 domains, which shows that the oligosaccharide moieties influence the opening of the Fc-fragment (Figure 2(a) and (b); Table 2). Interestingly in the shortest glycoform $(MN2F)_2$ the Cγ2–Cγ2 distance is reduced by 2.3 Å relative to the glycoform $(M3N2F)_2$ which carries two additional branching mannose residues (Table 2). Therefore, the hexasaccharide chain, with the branching mannose residues, is particularly important in effecting the open conformation of the horseshoe-shaped IgG-Fc fragment. Furthermore the maximal Cγ2–Cγ2 distance in glycoform $(G2F)_2$ shows that addition of GlcNAc residues to each arm of the oligosaccharide further influences the open conformation.

The Man8 residues of chain A and chain B have been found in close contact with each other for all glycoforms having branched oligosaccharides in space group $P2_12_12_1$. The Man4–Man4 distance (see Table 2) is also very similar in all IgG-Fc glycoforms of space group $P2_12_12_1$. The constitutive oligosaccharide/oligosaccharide contact can be maintained upon the opening of the protein moiety of the IgG-Fc fragment because movement of the oligosaccharides on the protein surface is allowed by the rearrangement of multiple but weak oligosaccharide/protein interactions (Figures 3(a) and 4). Taken together our observations indicate that oligosaccharide moieties serve as spacers holding both Cγ2 domains apart.

If the Cγ2 domains deviate largely from their symmetric positions, as in the native IgG-Fc crystals obtained under high salt conditions, the oligosaccharide/oligosaccharide interactions reorganise and in the case of $(MN2F)_2$ and $(G2F)_2$ ($C222_1$) are completely absent (data not shown). Although these findings may result from crystal contacts, which favour the observed IgG-Fc conformation, they may also be indicative of Cγ2 domain flexibility.

Although no destabilisation of the Cγ2 domains was observed upon removal of Man5 and Man8, as shown by differential scanning calorimetry (DSC), the affinity of the IgG-Fc fragment towards FcγRIIb was markedly decreased.[21] These findings, together with our structural data, lead to the conclusion that a closer approach of the Cγ2 domains interferes with effective FcγR binding. We emphasize, therefore, the role of Man8 as the contact sugar residue between the two oligosaccharide moieties at the Cγ2–Cγ2 interface, which defines a minimal Cγ2–Cγ2 distance. Although Man5 does not contribute to oligosaccharide/oligosaccharide interactions it may stabilise the oligosaccharide arrangement *via* its interaction with the Cγ2 domain and therefore also contributes to an effective FcγR–IgG-Fc complex formation.

The observed abrogation of receptor binding for the deglycosylated IgG-Fc fragment[21] may be due, partially, to an even more closed conformation of the IgG-Fc and, more importantly, to the lack of stabilising oligosaccharide/protein interactions resulting in an increased internal disorder of the Cγ2 domains. Evidence for this comes from the inability to crystallise the deglycosylated IgG-Fc fragment and its increased sensitivity to many proteases.[17]

Conformational changes in Cγ2 domains upon truncation of the oligosaccharide chain

The absence of GlcNAc6/GlcNAc9, i.e. in glycoforms $(M3N2F)_2$ and $(MN2F)_2$ (Figure 1), causes a shift of the entire oligosaccharide moiety accompanied by a synchronous movement of the C′E-loop compared to IgG-Fc structures containing residue GlcNAc6 in the α(1–6) arm (Figure 3(a) and (b)).

Interestingly structural changes of the C′E-loop are not identical in chain A and B, indicating that the C′E-loop gains more flexibility upon stepwise oligosaccharide truncation. We interpret this to be due to different crystal contacts in Cγ2A and Cγ2B domains that result in the "softened" C′E-loop assuming different conformations. The observed structural changes in the C′E-loop have an influence on the affinity of IgG-Fc for FcγRs as the C′E-loop is directly involved in IgG-Fc–FcγRIII contact.[22]

The results of isothermal titration calorimetry (ITC) and BIAcore experiments correlate well with the present observations. Mimura *et al*.[21] observed a slight reduction in affinity of the IgG-Fc $(M3N2F)_2$ glycoform for FcγRIIb, which was attributed to an unfavourable change in entropy. The binding of the "softer" and conformationally unrestrained IgG-Fc glycoforms to FcγRIIb seems to result in a reduction of conformational freedom, leading to an unfavourable entropy and finally to a lower affinity for the Fcγ-receptor. Our results suggest that the higher FcγRIIb affinity of all glycoforms containing GlcNAc sugar residues on the α(1–6) and α(1–3) arms of the oligosaccharide results from a rigid C′E-loop conformation, which does not change upon FcγR binding.

The observed conformational changes in the C′E-loop and the oligosaccharide on oligosaccharide truncation agree with ^{1}H NMR data obtained for the deglycosylated IgG-Fc fragment. These measurements indicate a structural change in the vicinity of the reporter group His268,[24,25] which is located in the BC-loop close to the N-glycosylation site at Asn297 (Figure 3(a)).

Carbohydrate–protein contacts

Multiple non-covalent sugar–protein interactions (Table 4 of Supplementary Materials) between the primary sugars GlcNAc1, GlcNAc3 and Man4 and sugars of the α(1–6) arm together with the *N*-glycosidic bond to Asn297 are responsible for the association of the oligosaccharide

with the protein surface. The superposition of Cγ2 domains of the two crystal forms of the $(G2F)_2$ glycoform reveals large conformational differences for sugars of the α(1–6) arm (Figure 4). Thus, the Gal7 and Man5 residues of the $(G2F)_2$ ($C222_1$) glycoform adopt a protein proximal position coplanar with the protein surface whilst they appear to be rotated by 90° and adopt a protein distal position in the structure of $(G2F)_2$ ($P2_12_12_1$) glycoform. Only GlcNAc6 does not lose protein contact in the solved IgG-Fc glycoform structures and shields the hydrophobic residue Phe243 from the solvent. In the $(M3N2F)_2$ glycoform, missing GlcNAc6, we find ordered water molecules around Phe243 which results in an unfavourable entropy (data not shown).

Although Man5 is present in the $(M3N2F)_2$ glycoform the rotation of the sugar plane by 90° prevents an effective contact to the protein surface. Therefore the situation in terms of thermal stability of the Cγ2 domain is very similar for the $(M3N2F)_2$ and $(MN2F)_2$ glycoforms, as shown by DSC experiments.[21]

A major decrease in melting temperature and unfolding enthalpy of the Cγ2 domains has been observed for the $(M3N2F)_2$ glycoform which lacks GlcNAc6, GlcNAc9 and later sugar residues[21] (Figure 1). This confirms our finding that GlcNAc6 stabilises the Cγ2 domain.

The inverse relation between the length of the oligosaccharide chain and the average *B*-factors of the oligosaccharide moieties and the C′E-loops (Table 3) is clearly due to the loss of multiple weak interactions which stabilise the oligosaccharide/protein contacts and rigidify the Cγ2 domains.

Conclusions

The results obtained from the structural investigation, reported here, together with data from thermodynamic and BIAcore experiments[21] lead us to propose that the stepwise decrease in K_A upon oligosaccharide truncation can be explained by both a decreased k_{on} and an increased k_{off}. Two independent observations support this theory. The "closed" conformations of glycoforms containing a truncated oligosaccharide tree need to undergo conformational rearrangement prior to FcγR binding, resulting in a reduced k_{on}, whereas an "open" IgG-Fc structure is the most favoured conformation for interaction with FcγRIII, as seen in the IgG-Fc–FcγRIII complex. Furthermore, truncation of the oligosaccharide results in the flexible C′E-loop adopting a more rigid and entropically unfavourable conformation during FcγR receptor binding, leading to both a decreased k_{on} and an increased k_{off} rate. This results in an overall decreased association constant $K_A = (k_{on}/k_{off})$. Further BIAcore experiments on FcγR-Fc binding could determine k_{on} and k_{off} rates separately to illuminate this question in detail.

Structural and thermodynamic data have shown that conformation and functionality of antibodies can be modulated by manipulation of their oligosaccharides. Glyco-engineered antibodies could up or down regulate the activity of effector cells by modulation of antibody affinity for Fcγ-receptors. Mammalian cell lines can be used for the production of recombinant chimeric and humanised IgG that express effector activities. However, cell engineering, with the introduction or deletion of genes coding for oligosaccharide modifying enzymes predetermined glycoforms of antibodies can be produced.

A glycoengineered anti-neuroblastoma chimeric IgG1 (chCE7) has been produced in Chinese hamster ovary (CHO) cells by the transfection of the β(1-4)-*N*-acetylglucosaminyltransferase III gene coding for an enzyme adding a bisecting GlucNAc sugar between the α(1–3) and the α(1–6) oligosaccharide arms.[26] The resulting antibody glycoform allows for the killing of neuroblastoma cells at ten- to 20-fold lower concentrations than IgG glycoforms lacking the bisecting GlucNAc. Recently therapeutic applications for aglycosylated anti-CD3 antibodies have been proposed, since they induce immune suppression, have a long half life compared to Fab or $F(ab')_2$ fragments, but do not trigger "first-dose" side effects, probably due to their inability to bind and activate FcγRs.[27,28]

As the IgG-Fc–FcγR interaction is strongly influenced by the IgG-Fc glycosylation profile, modulation of the effector functions of therapeutic antibodies may be achieved by selection of an appropriate glycoform. However, so far it is still unclear how the glycosylation pattern of the IgG-Fc portion can be rationally influenced to modulate the effector functions of therapeutic antibodies, which would be of major interest. The present structural data should contribute to a better understanding of the mechanism by which the oligosaccharide influences the structure and function of IgG antibodies.

Data from mutational screening of all solvent-exposed amino acid side-chains in Cγ2 and Cγ3 domains of human IgG1-Fc suggests that residues distant from the receptor–IgG-Fc interface can influence IgG-Fc affinity for FcγRs.[29] For instance the mutation of Glu430 to Ala in the Cγ2–Cγ3 cleft results in an improved binding of FcγRIIb to IgG1-Fc. This effect might be caused by the fixation of the Cγ2–Cγ3 joint to an angle similar to that found in the bound state of the Fc-fragment. Thus, in combination protein evolution and glycoengineering could provide a means for generating panels of antibodies with unique combinations of biological activities.

Materials and Methods

Proteins

Human IgG1 (Cri, allotype G1m(f)) was isolated from serum of a patient with multiple myeloma. The IgG1-Fc

IPR2025-00604
Patent Owner's Exhibit 2023
Page 000009

fragment was generated by papain digestion.[11] The protocols which were used to generate the glycoforms $(G0F)_2$, $(M3N2F)_2$, and $(MN2F)_2$ enzymatically have previously been described.[21] Glycoform $(G2F)_2$ was prepared with sialidase (*Arthrobacter ureafaciens*, Roche, 0.1 unit/mg of Fc) in 50 mM acetate buffer (pH 5.0) at 37 °C for 24 hours and subsequently with galactosyltransferase (bovine milk, Oxford Glycosciences, 0.12 unit/mg of Fc) in 20 mM Hepes, 0.15 M NaCl, 5 mM $MnCl_2$, 13 mM UDP-Gal (pH 7.0) at 37 °C for 24 hours. The protein was affinity purified using a protein G-Sepharose 4B column.

The nomenclature which was used for IgG-Fc glycoforms is as follows: $(G2F)_2$: biantennary digalactosylated and fucosylated Fc glycoform, previously abbreviated as $(NA2F)_2$;[20,21] $(G0F)_2$: biantennary non-galactosylated and fucosylated Fc glycoform, previously as $(NGA2F)_2$; $(M3N2F)_2$: Fc with fucosylated N-linked pentasaccharide cores; $(MN2F)_2$: Fc with fucosylated N-linked trisaccharide cores. G, M, N and F denote galactose, mannose, GlcNAc and fucose residues, respectively.

Complete deglycosylation of 4 mg native Fc-fragment was achieved by PNgase F digestion (50 units, Roche) at 37 °C for 60 hours. Subsequently the completely deglycosylated Fc was purified by size exclusion chromatography (Superdex 75, PharmaciaAmersham) in PBS.

Crystallisation of Fc glycoforms

For crystallisation trials 5 μl of the Fc-fragment solutions (10 mg/ml) were dialysed against 20 ml of 20 mM Mes/TRIS buffer at pH 6, 6.5, 7.0, 7.5, 8.0 and 9.0 or water at 4 °C and yielded crystals within two days which were used for data collection. Crystals of native Fc-fragment under high salt conditions (native high salt) were grown in space group $P6_122$ within six months at room temperature in sitting drops using 2 μl of protein and 1 μl of reservoir solution (0.1 M Na/acetic acid (pH 4.5), 2 M NaCl).

Diffraction data collection and data reduction

Cryoprotection was achieved by soaking the crystals successively in water containing 10% and 15% 2,3-butanediol, respectively. For Fc-glycoform $(G0F)_2$ 20% glycerol in water was used for cryoprotection. From accordingly treated crystals at a temperature of 100 K diffraction data was collected at a wavelength of 1.5418 Å on an image plate system (MAR research) using graphite monochromated Cu Kα radiation from a RU200b rotating anode generator (Rigaku) operated at 50 kV and 100 mA (Table 1). All data were indexed, integrated and scaled using the program DENZO and SCALEPACK[30] and further processed and reduced with routines of the ccp4 package.[31]

Structure determination

The Fc-fragment structures were solved by molecular replacement using the program AmoRE.[32] During refinement a rigid body routine was employed using the Fc domains as individual rigid domains followed by an energy minimisation of the atomic coordinates and *B*-factor minimisation.

For both structures of 2.4 Å resolution an atomic energy minimisation in conjunction with individual *B*-factor refinement was performed. All other structures of lower resolution were refined by two cycles of energy minimisation refinement followed by a domain-wise *B*-factor refinement.

Corrections to the model deduced from the map were applied with the model building program MAIN.[33] After rebuilding of the structure another cycle of energy minimisation and *B*-factor refinement was performed.

The dihedrals of all amino acid residues were refined to allowed values in the Ramachandran plot. The model was further improved in successive cycles of model building and refinement. Superimposition of IgG1-Fc structures was performed with the program LSQMAN.[34]

Protein Data Bank accession number

The coordinates and structure factors of the Fc glycoforms $(MN2F)_2$, $(M3N2F)_2$, $(G0F)_2$, $(G2F)_2$ (in space group $P2_12_12_1$ and $C222_1$) and native Fc crystallised at high salt conditions have been deposited in the RCSB Protein Data Bank under the accession numbers 1H3T, 1H3U, 1H3X, 1H3V, 1H3W and 1H3Y.

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