



The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms

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Abstract

Antibodies are multifunctional molecules that following the formation of antibody antigen complexes, may activate mechanisms to effect the clearance and destruction of the antigen (pathogen). The IgG molecule is comprised of three globular protein moieties (2Fab + Fc) linked through a flexible hinge region. While the Fabs bind antigens, the Fc triggers effector mechanisms through interactions with specific ligands, e.g. cellular receptors (FcγR), and the C1 component of complement. Glycosylation of IgG-Fc has been shown to be essential for efficient activation of FcγR and C1. We report the generation of a series of truncated glycoforms of IgG-Fc, and the analysis of the contribution of the residual oligosaccharide to IgG-Fc function and thermal stability. Differential scanning microcalorimetry has been used to compare the stabilities of the homogeneous glycoforms of IgG1-Fc. The results show that all truncated oligosaccharides confer a degree of functional activity, and thermodynamic stability to the IgG1-Fc, in comparison with deglycosylated IgG1-Fc. The same truncated glycoforms of an intact IgG1 anti-MHC Class II antibody are shown to exhibit differential functional activity for FcγRI and C1 ligands, relative to deglycosylated IgG1. The minimal glycoform investigated had a trisaccharide attached to each heavy chain and can be expected to influence protein structure primarily in the proximity of the N-terminal region of the C_H2 domain, implicated as a binding site for multiple effector ligands. These data provide a thermodynamic rationale for the modulation of antibody effector functions by different glycoforms. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The IgG molecule is a multifunctional glycoprotein comprised of three ‘independent’ protein moieties connected thorough a flexible linker (the ‘hinge’). Two protein moieties express an antigen specific binding site (IgG-Fab) whilst the third, IgG-Fc, expresses interaction sites for ligands that activate clearance

mechanisms. The IgG-Fc is a homodimer comprised of inter-chain disulphide bonded hinge regions, glycosylated C_H2 domains, and non-covalently paired C_H3 domains (Burton and Woof, 1992; Harris et al., 1997; Jefferis et al., 1998). Indirect approach suggests that interaction sites for the cellular Fc receptors (FcγRI, FcγRII, FcγRIII) are localised to the hinge proximal region of the C_H2 domain (Canfield and Morrison, 1991; Lund et al., 1991; Sarmay et al., 1992; Jefferis et al., 1998). Direct evidence has now been provided from the crystal complex of human IgG1-Fc with recombinant human FcγRIII receptor (Sondermann et al., 2000), and NMR studies of the interaction of

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mouse IgG2b-Fc with mouse Fc γ RII (Kato et al., 2000). Indirect approach also suggests, that the interaction site on human IgG1 for the C1q component of complement is similarly localised to the hinge proximal region (Morgan et al., 1995; Xu et al., 2000), in contrast to the mouse IgG2b for which structure more proximal to the C_H2/C_H3 interface was reported to be influential (Duncan and Winter, 1988). Monomeric IgG is univalent for these ligands, however, the formation of IgG-antigen immune complexes result in the generation of multi-valent aggregated IgG-Fc facilitating effector ligand cross-linking, and consequent activation. Glycosylation of the IgG-Fc is essential for the activation of these effector ligands (Pound et al., 1993; Lund et al., 1996; reviewed in Jefferis et al., 1998). In contrast the X-ray crystallographic analysis of IgG-Fc in complex with the neonatal Fc receptor (Fc γ Rn) (Burmeister et al., 1994), rheumatoid factor (RF) autoantibody (Corper et al., 1997), Staphylococcal protein A (SpA) (Deisenhofer, 1981), and Streptococcal protein G (SpG) (Sauer-Eriksson et al., 1995) have revealed overlapping non-identical interaction sites at the inter C_H2/C_H3 domain region that are minimally affected by glycosylation status.

Crystal structures of IgG-Fc reveal a distinct conformation for the oligosaccharide resulting from multiple non-covalent interactions with the protein, such that each has a reciprocal influence on the conformation of the other (Deisenhofer, 1981; Padlan, 1990; Burmeister et al., 1994; Sauer-Eriksson et al., 1995; Corper et al., 1997). The oligosaccharide appears to be sequestered within the “internal” space of the C_H2 domain (Fig. 1). The oligosaccharide moiety is of the complex biantennary type, and exhibits heterogeneity with respect to the terminal sugars attached (Jefferis et al., 1990, 1998). In the present study, we have generated homogeneous naturally occurring glycoforms and also truncated glycoforms of an intact humanised IgG1 anti-MHC class II antibody, and IgG1-Fc fragments in order to investigate the influence of the various oligosaccharides on structural stability and effector functions. Differential scanning microcalorimetry (DSC) has been applied to determination of the thermal unfolding temperatures (T_m) of the C_H2 and C_H3 domains, as an index of stability. Classical pathway activation of complement, and Fc γ RI activation have been used as indices of functional activity. We demonstrate an incremental increase in IgG-Fc stability and biological activity for all glycoforms relative to that of the deglycosylated molecule and that the presence of the trisaccharide MN2 confers both structural stability and functional activity on its (MN2)₂ glycoform. These data support the involvement of the hinge proximal region of IgG-Fc in Fc γ R and C1 recognition and activation.

2. Materials and methods

2.1. Proteins

The monoclonal protein IgG1-Wid was isolated from a myeloma serum and the IgG1-Fc fragment generated as reported earlier (Jefferis et al., 1990). The humanised recombinant L243-IgG1 protein was purified from culture supernatant by affinity chromatography on a Staphylococcal protein A-Sepharose 4B matrix, as reported earlier (Morgan et al., 1995). The protocol for generating the truncated glycoforms is illustrated for protein IgG1-Fc Wid; (NGA2)₂ glycoforms, native IgG1-Fc (1 mg) in 50 mM acetate buffer, pH 5.0, was exposed to sialidase (300 mU; *Arthrobacter ureafaciens*, Boehringer Mannheim) at 37°C for 24 h. The pH was adjusted to 6.0, and β -galactosidase (120 mU; *Diplococcus pneumoniae*, Boehringer Mannheim) added and the solution incubated at 37°C for 24 h. (M3N2)₂ glyco-

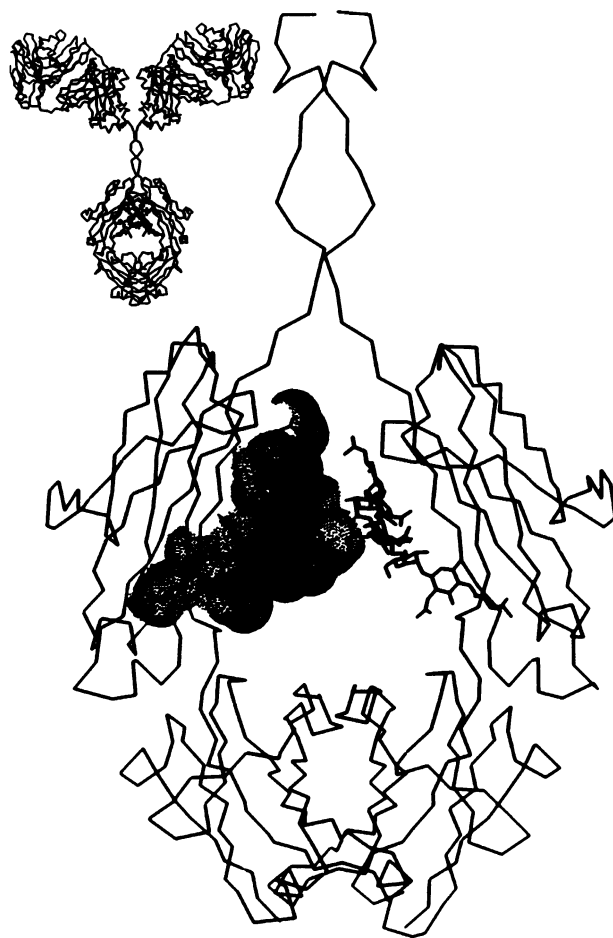


Fig. 1. Alpha carbon backbone structure of IgG-Fc showing the oligosaccharide moieties attached to the C_H2 domains. The left hand domain shows the van der Waals surface of an oligosaccharide comprised of the NGA2 core (Fig. 2A-ii) bearing fucose on the primary *N*-acetylglucosamine and galactose on the α (1→6) arm. Inset, the complete IgG molecule. Adapted from Lund et al. (1991).

forms: (NGA2)₂ IgG1-Fc (1 mg), in 50 mM citric acid-Na₂HPO₄, pH 4.8, was exposed to *N*-acetyl- β -D-glucosaminidase (180 mU, *Diplococcus pneumoniae*, Boehringer Mannheim) at 37°C for 24 h. (MN2)₂ glycoforms, (M3N2)₂ IgG1-Fc (1 mg) in 50 mM acetate buffer containing zinc acetate (2 mM), pH 5.0, was exposed to α -mannosidase (Jack bean, Oxford Glyco-science) at 37°C for 24 h. Deglycosylated IgG1-Fc: native IgG1-Fc (1 mg) in 40 mM KH₂PO₄, 10 mM EDTA, pH 7.4 was exposed to peptide-*N*-glycosidase F (6U; *Flavobacterium meningosepticum*, Boehringer Mannheim) at 37°C for 72 h. Following the exposure to glycosidases all proteins were affinity purified from a Streptococcal protein G Sepharose 4B (Pharmacia) column, eluted with 0.1 M glycine-HCl buffer, pH 2.7. Eluates were immediately neutralised by the addition of 1 M Tris/HCl, pH 9.0, and dialysed extensively against PBS. The homogeneous glycoforms of L243-IgG1 were prepared in the same manner using one-third of the quantities of glycosidases used for IgG1-Fc Wid, thus maintaining the same molar ratios.

2.2. Electrospray ionisation mass spectrometry (ESI-MS)

ESI-MS was performed using an LCT instrument (Micromass, Manchester, UK), and MassLynx data acquisition. This instrument is an orthogonal acceleration time-of-flight mass spectrometer, with a Z-spray electrospray ion source. The mass spectrometer was operated in the positive-ion mode. Resolution of the mass spectrometer was 5000 at *m/z* 1500. The cone voltage was set to 35 V. The purified IgG1-Fc Wid proteins (1 mg ml⁻¹) were extensively dialysed against distilled water, and made 1% in formic acid; the L243-IgG1 proteins were dialysed against 4.5% acetic acid extensively after reduction with 10 mM dithiothreitol for 1 h. Ten microliters of the sample solution was injected via a Rheodyne valve into the mobile phase of 50% acetonitrile, and 50% of 0.1% formic acid in water. Between 10 to 20 spectra were averaged, baseline subtracted and deconvoluted using MAX.ENT. software.

2.3. Differential scanning microcalorimetry (DSC)

Experiments were performed on a VP-DSC microcalorimeter (MicroCal Inc, Northampton, MA, USA) with IgG1-Fc sample concentrations of ~4 μ M and a scan rate of 60°C h⁻¹. No concentration dependence of the thermal unfolding temperatures (*T*_m), and calorimetric enthalpies (ΔH) were noted at this low concentration. Data were collected, and analysed using ORIGIN 4.1 software, essentially as described earlier (Ghirlando et al., 1999). The area below each peak corresponds to ΔH , and van't Hoff enthalpy (ΔH_v) was calculated using the formula,

$$\Delta H_v = 4RT_m^2 \langle \Delta C_p \rangle_{tr, max} / \Delta H$$

where, $\langle \Delta C_p \rangle_{tr, max}$ is the measured heat capacity at the peak maximum, *R* is gas constant (1.987 cal mol⁻¹ K⁻¹). The cooperativity of the heat transition was evaluated from the ratio $\Delta H / \Delta H_v$ (Sturtevant, 1987).

The free energy of stabilization provided by the truncated oligosaccharide chains was estimated using the following equation (Brandts et al., 1989),

$$\Delta G = \Delta H^d (1 - T_m^* / T_m^d)$$

where, ΔH^d is ΔH for the unfolding of deglycosylated C_{H2} domain, *T*_m^d is *T*_m (in K) of the deglycosylated C_{H2} domains, *T*_m^{*} is *T*_m of the C_{H2} domains of an individual glycoform. ΔG is for the temperature *T*_m^{*}. The reference state ($\Delta G = 0$) corresponds to the state where the oligosaccharides are separated and/or the C_{H2} domains melt.

2.4. Superoxide assays

2.4.1. Activation of U937 cells by JY cells sensitised with homogeneous glycoforms of L243-IgG1

Superoxide anion production was measured as lucigenin-enhanced chemiluminescence, essentially as detailed earlier (Pound et al. 1993). JY and U937 cells were maintained in RPMI1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum (HIFCS), 2 mM glutamine, 200 IU ml⁻¹ penicillin, and 100 μ g ml⁻¹ streptomycin (10% RPMI). To induce differentiation and the capacity to generate superoxide the U937 cells were cultured in the presence of IFN γ (1000 units ml⁻¹, Janssen Biochimica, Beerse, Belgium) for 2 days. The cells were washed and resuspended at 2 $\times$ 10⁶ cells ml⁻¹, in Hanks balanced salt solution without phenol red (Gibco BRL, UK), and containing 20 mM HEPES and BSA (0.15 mM; γ globulin free — Sigma, Dorset, UK), pH 7.4 (HBSS-BSA). JY cells (2.5 $\times$ 10⁷ ml⁻¹) were sensitised by the addition of various glycoforms of L243-IgG1 antibody (0.6–10 nM), and incubation at 37°C for 30 min. The cells were washed three times with PBS, and resuspended at 2.5 $\times$ 10⁷ cells ml⁻¹ in HBSS-BSA. The U937 cells (100 μ l, 2 $\times$ 10⁶ ml⁻¹) were added to plastic tubes, and lucigenin (20 μ l, 2.5 mM) added and the tubes were warmed in a 37°C water bath for 5 min. The sensitised cells (80 μ l, 2.5 $\times$ 10⁷ ml⁻¹) were added, and the chemiluminescence response was monitored at 37°C over a 30 min period using a Berthold LB953 luminometer.

2.4.2. Inhibition of native L243-IgG1 by homogeneous glycoforms of IgG1-Fc Wid

Recognition of Fc γ RI by homogeneous IgG1-Fc glycoforms was determined by inhibition of the assay described above. The IgG-Fc glycoforms (20 μ l, 3–5000 nM) were added to U937 cells (80 μ l, 2.5 $\times$ 10⁶ cells

ml⁻¹) containing Lucigenin (20 µl, 2.5 mM in HBSS) in plastic tubes, which were warmed in a 37°C water bath for 5 min. The JY cells (80 µl, 2.5 × 10⁷ cells ml⁻¹), sensitised with L243-IgG1 (13 nM), were added, and the chemiluminescence monitored at 37°C for 30 min using an LB953 Berthold luminometer. The IC₅₀ values were determined and relative inhibitory activity is reported as % of the control value obtained in the absence of inhibitor; unsensitised JY cells were included as a negative control.

2.4.3. Complement-mediated lysis

Target JY cells were washed once in 10% RPMI and the cell density adjusted to 5 × 10⁶ cells ml⁻¹ in 15% RPMI containing probenecid (*p*; 0.5 mM Wallac, Finland) (15% RPMI + *p*). Aliquots (1 ml) of cells were added to wells in a Limbro plate and labelled with fluorescence enhancing ligand BATDA (bis(acetoxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate) at 10 µM and sensitised with L243-IgG1 antibody (0.5–16 nM) at 37°C for 30 min with gentle shaking. After washing 3 × with 15% RPMI + *p* the labelled and sensitised cells (100 µl, 5 × 10⁵ cells ml⁻¹) were added to the wells of a U shaped microtitre plate. Fresh human serum at 50% (100 µl), diluted in 15% RPMI + *p* medium, was added to each well. Control wells included maximum release with TritonX-100 (0.1%) and spontaneous release in the absence of serum. The plate was centrifuged at 1800 rpm for 5 min. to pellet the cells, and incubated at 37°C for 1 h in a humidified, 5% CO₂ atmosphere. The plate was centrifuged again and 20 µl of supernatant was transferred to wells of a "low-background" plate (Wallac). Europium solution (200 µl, Wallac) was added to each well and the plate shaken for 15 min. Fluorescence was measured using a time-resolved fluorometer (DELFI, Wallac).

3. Results

3.1. Homogeneous glycoforms of IgG and IgG-Fc

Electrospray ionisation mass spectrometry (ESI-MS) of deglycosylated IgG1-Fc (Wid) gave a mass of 49 871 Da, in agreement with the calculated mass (49 866 Da) for the IgG1-Fc protein (allotype G1m(z)) in which, asparagines 297 were replaced with aspartic acid, following deglycosylation, and lacking the C-terminal lysine residue of native IgG. The ESI-MS profile of the native IgG1-Fc-Wid (Fig. 2A-i) reflected the glycosylation heterogeneity previously reported (Jefferis et al., 1990). The structure of the homogeneous IgG1-Fc glycoforms was confirmed by mass analysis (Fig. 2A, ii, iii, iv, v and Table 1). Homogeneous glycoforms of the recombinant humanised L243-IgG1 antibody were generated by exposure to glycosidases, as for IgG1-Fc, and

the structures confirmed by ESI-MS after mild reduction and acidification, on the basis of the mass of deglycosylated H chain (49 372 Da, Fig. 2B-v), and the calculated masses of the truncated oligosaccharide chains (Table 1).

3.2. Differential scanning microcalorimetry

Thermograms for each of the glycoforms of IgG1-Fc (Wid) (Fig. 3) exhibit two thermal transitions. The lower temperature transition (*T*_{m1}) is due to the thermal unfolding of the C_H2 domain, and the second transition the unfolding of the C_H3 domain (Tishchenko et al., 1998; Tishchenko et al., 1998; Ghirlando et al., 1999). The thermodynamic parameters, based on a two-state model for each transition, describing these transitions are shown in Table 2. It is evident that the transition temperature of the C_H2 domain is dependent on the glycoform analysed whilst *T*_{m2} of the C_H3 domain is glycoform independent. No difference in *T*_{m1} is detected between the native and the degalactosylated (NGA2)₂ glycoforms, indicating that the terminal galactose moieties do not stabilise the C_H2 domain to a significant extent. However, we have previously shown that differential galactosylation of the IgG4-Fc influences ΔH (Ghirlando et al., 1999).

Removal of terminal *N*-acetylglucosamine residues resulted in a significant reduction in stability of the C_H2 domain, as evidenced by a decrease in *T*_{m1} by 3.7°C and ΔH for the (M3N2)₂ glycoform (Table 2). No measurable differences in ΔH 1 were observed between the (M3N2)₂ and (MN2)₂ glycoforms. There is a significant difference, however, in the *T*_{m1} of the deglycosylated IgG1-Fc, relative to the (MN2)₂ glycoform (1.4°C). This decreased *T*_{m1} value shows that the native oligosaccharides provide a maximum of -1.8 kcal mol⁻¹ of free energy stabilisation. The (MN2)₂ tri-saccharides are sufficient to provide, at most -0.5 kcal mol⁻¹ of stabilisation. In addition, the ratio ΔH 1/ ΔH v1 of the deglycosylated IgG1-Fc increases to 1.4 indicating that the C_H2 domains form an unfolded intermediate in the process of thermal denaturation (Sturtevant, 1987), and no longer exhibit a two-state cooperativity, unlike the C_H2 domains of the other glycoforms that behave as a single cooperative unit.

3.3. Superoxide generation by U937 cells activated through FcγRI by L243-IgG1 sensitised JY cells

3.3.1. Activation of U937 cells by L243-IgG1 sensitised JY cells

The native L243-IgG1 anti-MHC class II antibody was glycosylated with a fucose residue attached to the primary *N*-acetylglucosamine residue (Fig. 2B). The MHC class II expressing JY cell line was sensitised with native and each of the truncated, homogenous glyco-

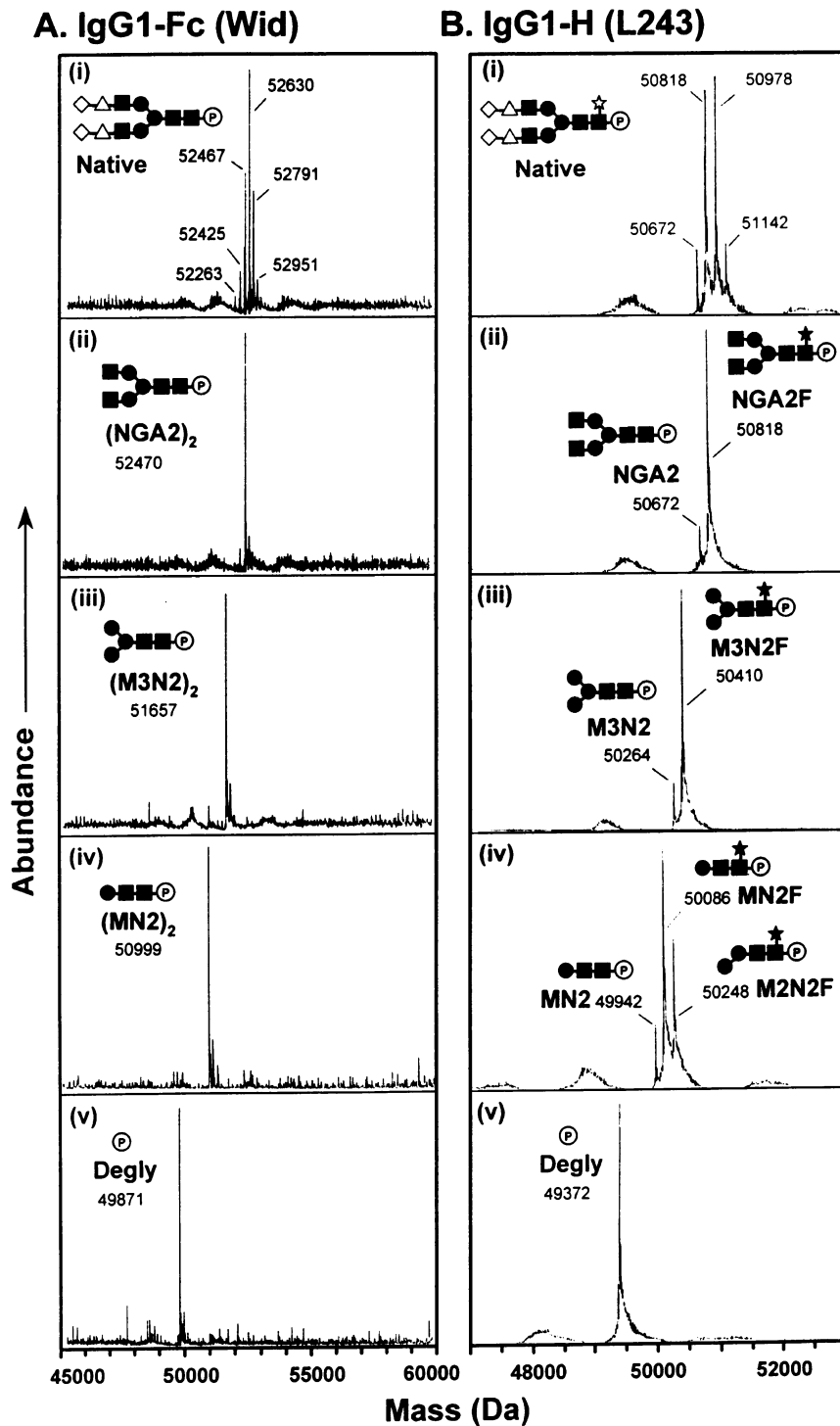


Fig. 2. The mass and homogeneity of homogeneous glycoforms of the Fc fragment of IgG1 Wid (A) and the H chain of L243-IgG1 (B) were determined by ESI-MS, (i) native protein, (ii) sialidase- and β -galactosidase-treated protein, (iii) *N*-acetylglucosaminidase-treated protein, (iv) α -mannosidase-treated protein, (v) peptide-*N*-glycosidase F-treated protein. Degly refers to deglycosylated protein. Open symbols in panels (i) represent variable attachment of terminal fucose, galactose and sialic acid residues; The sugars are represented as follows, *N*-acetylglucosamine, N or the symbol ■; mannose, M or ●; △, galactose; ◇, sialic acid; p, protein.

forms of L243-IgG1, and their ability to activate U937 cells was compared to that of deglycosylated L243-IgG1. The results show no significant difference in Fc γ RI activating capacity between the native and

(NGA2F)₂ glycoforms. However, (M3N2F)₂ glycoform showed a slight reduction in activity. The activity was further compromised for the (MN2F)₂ glycoform, the response at 10 nM of L243-IgG1 being ~50% of that

obtained for higher glycoforms (Fig. 4A). This activity is appreciable in comparison to that obtained for the deglycosylated protein, ~ 30% of that obtained for the higher glycoforms. Proteins subjected to “mock” digestion served as controls, and showed that exposure to the incubation buffers for the glycosidase treatments does not have adverse effects on the functional activities of the proteins (inset of Fig. 4A).

3.3.2. Inhibition of native L243-IgG1 by homogeneous glycoforms of IgG-Fc

The glycoforms of IgG1-Fc (Wid) generated for the calorimetric experiments were used to determine the IC₅₀ for inhibition of the superoxide response of U937 cells elicited by JY cells sensitised with native L243 IgG1 (Fig. 4B). The values obtained for native IgG1-Fc, (NGA2)₂, (M3N2)₂, (MN2)₂ and deglycosylated glycoforms were 4.4, 4.8, 5.2, 7.6 and 170 nM, respectively. This corresponds to the relative inhibitory values of 100, 92, 85, 58 and 3%, respectively.

3.4. Complement-mediated lysis of L243-IgG1 sensitised JY cells

The complement-mediated lysis of BATDA-loaded, L243-IgG1-sensitised JY cells was performed. Maximum lytic activity was achieved for native L243-IgG1 and the homogeneous (NGA2F)₂ glycoform whilst no lysis was observed for the deglycosylated protein (Fig. 5). The (M3N2F)₂ and (MN2F)₂ glycoforms exhibited residual activity but required a ~ 10 fold higher level of sensitisation to achieve the same level of lysis as for native protein.

4. Discussion

Crystal structures of human IgG-Fc reveal a distinct conformation for the oligosaccharide. This is unusual for glycoproteins and results from multiple non-covalent interactions between the oligosaccharide and the protein such that each has a reciprocal influence on the conformation of the other (Deisenhofer, 1981; Padlan, 1990; Burmeister et al., 1994; Sauer-Eriksson et al., 1995; Corper et al., 1997). The thermodynamic parameters describing the unfolding of IgG-Fc (Table 2) reflect these interactions, with the thermal unfolding of the C_H2 domains showing a progressive reduction in stability with loss of sugar-protein interactions whilst the unfolding of the C_H3 domain is unaffected. These properties of IgG-Fc contrast with those observed for other glycoproteins bearing a single N-linked oligosaccharide in which the oligosaccharide is mobile, and does not confer thermal stability, e.g. chicken avidin and ova-transferrin (Wang et al. 1996).

The thermodynamic parameters describing C_H2 thermal denaturation of all glycosylated IgG-Fc are consistent with a cooperative unfolding, however, for deglycosylated IgG1-Fc these parameters are interpreted as indicative of a non-cooperative unfolding, involving at least a single intermediate. We propose that this intermediate is a partially unfolded C_H2 domain pair possessing hinge proximal disordered/unfolded loops. Although the calorimetric data for the unfolding of the C_H2 domains incorporates contributions from the unfolding of the C_H2 domains, loss of the C_H2/C_H3 inter-domain interactions, and C_H2/oligosaccharide contacts, the non-cooperativity of deg-

Table 1
Determination of the glycoforms by ESI-MS^a

IgG1-Fc (Wid)			IgG1-H (L243)		
Glycoform	Molecular mass (Da)		Glycoform	Molecular mass (Da)	
	Observed	Calculated		Observed	Calculated
Degly	49871	49866	Degly	49372	N.D.
(MN2) ₂	50999	51001	MN2	49942	49940
(M3N2) ₂	51657	51650	MN2F	50086	50086
NGA2+NM3N2	52263	52260	M2N2F	50248	50248
NA1+NM3N2	52425	52422	M3N2	50264	50265
(NGA2) ₂	52467	52463	M3N2F	50410	50411
NGA2+NA1	52630	52625	NGA2	50672	50671
NGA2+NA2/(NA1) ₂	52791	52788	NGA2F	50818	50817
NA1+NA2	52951	52950	NA1F	50978	50979
			NA2F	51142	51141

^a NM3N2, glycoform bearing hexasaccharide GlcNAc(Man)₃(GlcNAc)₂; NA1(F), non-fucosylated (fucosylated), monogalactosylated glycoform; NA2(F), non-fucosylated (fucosylated), digalactosylated glycoform. For the calculation of the masses of the glycoforms of IgG1-H (L243), Asp297 in deglycosylated Fc was replaced with Asn by subtracting 1 Da.

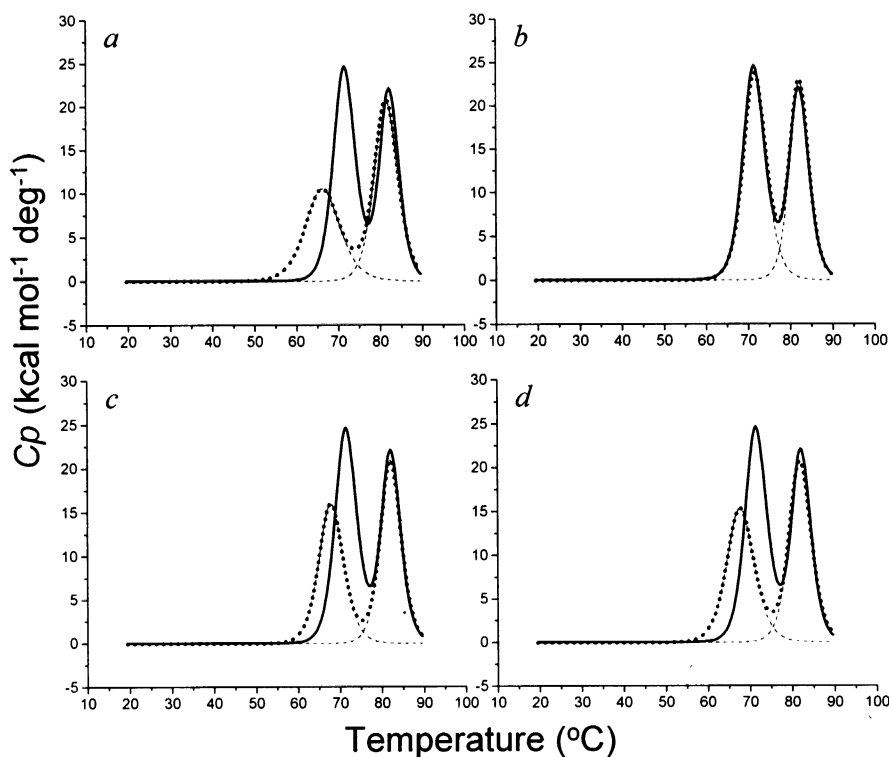


Fig. 3. Deconvoluted thermograms of native and (a) deglycosylated; (b) (NGA2)₂; (c) (M3N2)₂ and (d) (MN2)₂ glycoforms of IgG1-Fc Wid, in PBS, pH7.4. In each thermogram the solid line represents the profile for native IgG-Fc. The data were baseline corrected.

lycosylated C_H2 domain together with reduced T_m and ΔH indicate destabilisation of the domain structure, and may account for the compromised functional activities of deglycosylated IgG and IgG-Fc shown in Fig. 4 and Fig. 5.

We have proposed that the interactions between the protein and hinge proximal sugar residues result in the generation of a population of structural conformers; amongst which are specific conformers that recognise individual effector ligands (Jefferis et al., 1998). The demonstration of a degree of structural stability, and FcγRI functional activity conferred on IgG-Fc by the presence of the trisaccharide MN2 is consistent with this model. The anticipated non-covalent interactions between the three sugars, and amino acid residues may be sufficient to impose local conformational constraints on the protein structure (Fig. 1) (Deisenhofer, 1981; Padlan, 1990). This influence of the MN2 trisaccharide on structural stability of the C_H2 domain is consistent with the prediction that these three sugars have the potential to make a total of 31 contacts with 7 amino acid residues in the hinge proximal region (Padlan, 1990). For the L243 antibody 38 contacts with 8 amino acid residues are predicted, due to the presence of fucose on the primary *N*-acetylglucosamine residue. These interactions are critical for the generation of conformation at the hinge proximal region of the C_H2 domain compatible with FcγRI and C1 binding and

activation. Mobility of the hinge region within an intact IgG molecule has been observed for a mouse IgG2a protein; the authors concluding "...overall, the hinge, having an extent of 50 Å, is clearly very fluid and flexible, likely a continuum of conformations rather than a small, discrete set" (Harris et al., 1997). Similarly, an NMR study of the complex formed between mouse IgG2b, and soluble mouse FcγRII yielded a double peak for the hinge leucine 234 residues and this was interpreted to be consistent with each heavy chain leucine 234 oscillating between different conformations, or each adopting different conformations (Kato et al., 2000). It is now recognised that many proteins and protein domains are only partially folded or are unstructured under physiological conditions but become structured upon binding to their biological target (Dyson and Wright, 1998). These data would appear to be at variance with an NMR study that concluded that in the absence of terminal galactose residues the oligosaccharide moieties do not interact with the protein (Wormald et al., 1997) in which case it could not influence protein stability or conformation.

The influence of the MN2 trisaccharide on protein stability and human FcγRI, and complement activation demonstrated in this study strongly suggests that interaction sites of IgG for these ligands are located in the hinge proximal regions. Details as to how effector functions are mediated are gradually understood. Cellu-

Table 2
Thermodynamic parameters of homogenous glycoforms of human IgG1-Fc (Wid) by DSC

Glycoform	First transition				Second transition			
	T_{m1} (°C)	$\Delta H1$ (kcal/mol)	ΔH_{v1} (kcal/mol)	Ratio 1	T_{m2} (°C)	$\Delta H2$ (kcal/mol)	ΔH_{v2} (kcal/mol)	Ratio 2
Native	71.4 ± 0.06	158 ± 6	146 ± 4	1.1 ± 0.07	82.2 ± 0.03	126 ± 6	172 ± 4	0.7 ± 0.05
(NGA2) ₂	71.5 ± 0.04	155 ± 8	141 ± 3	1.1 ± 0.05	82.3 ± 0.05	125 ± 3	171 ± 4	0.7 ± 0.05
(M3N2) ₂	67.7 ± 0.06	113 ± 16	124 ± 7	0.9 ± 0.2	82.2 ± 0.04	117 ± 9	166 ± 6	0.7 ± 0.08
(MN2) ₂	67.3 ± 0.08	105 ± 3	124 ± 4	0.8 ± 0.05	82.1 ± 0.06	108 ± 2	168 ± 5	0.6 ± 0.03
Degly	65.9 ± 0.15	113 ± 7	83 ± 4	1.4 ± 0.15	81.5 ± 0.07	140 ± 9	140 ± 4	1.0 ± 0.09

lar FcγRs are activated following their cross-linking by divalent or multivalent IgG-Fc aggregates (immune complexes). Since FcγR bearing cells co-exist in the blood, and extra-vascular tissues spaces with relatively high concentrations of monomeric IgG, without activation, it follows that monomeric IgG must be functionally univalent for FcγR. This rationalisation has been vindicated in numerous studies (Koolwijk et al., 1989; Ghirlando et al., 1995; Haagen et al., 1995). However, extrapolations from recent crystal structures for soluble FcγRIIIa (Maxwell et al., 1999), and FcγRIIb (Sondermann et al., 1999) were used to generate conflicting models for the formation of IgG-Fc/FcR complexes. The FcγRIIIa molecule was seen to form dimers in the crystal, and it was suggested that these dimers generate a single IgG-Fc recognition site. Assuming interaction at the hinge proximal region, and engagement of both heavy chains results yielded an asymmetric (FcγRIIIa)₂-(IgG-Fc) complex in which the IgG is functionally monovalent. The model developed for a FcγRIIb/IgG complex was radically different since the interaction site for FcγRIIb was reasoned to be at the C_{H2}/C_{H3} interface and the IgG-Fc to be functionally divalent (Sondermann et al., 1999). The latter group has since published the X-ray crystal structure of a complex formed between soluble human FcγRIII and IgG1-Fc. The complex stoichiometry is 1:1, and the interaction site is shown to be at the hinge proximal region with lower hinge amino acid residues, and the primary N-acetylglucosamine sugar making contacts with the FcγRIII molecule (Sondermann et al., 2000). From homology consideration the authors proposed that interaction sites on other immunoglobulin isotypes for their FcR will be localised to a comparable region of the Ig molecule. Such a homologous binding site on human IgE has been identified from solution of the X-ray crystal structure of human IgE-Fc in complex with the human FcεRIα chain (Garman et al., 2000). Similarly, a study of mouse FcγRII binding to mouse IgG2b, in solution, has concluded that the stoichiometry is 1:1 and NMR data has been interpreted as providing direct evidence for a hinge proximal binding (Kato et al., 2000). We also confirmed a 1:1 stoichiometry for the interaction between human FcγRIIb and

IgG-Fc in solution by isothermal titration calorimetry (Mimura, et al., manuscript in preparation).

It is established that glycosylation of human IgG-Fc is essential for efficient activation of cellular Fcγ receptors and the classical pathway of complement activation. The structure of the oligosaccharide attached can also influence biological activity. It is well established

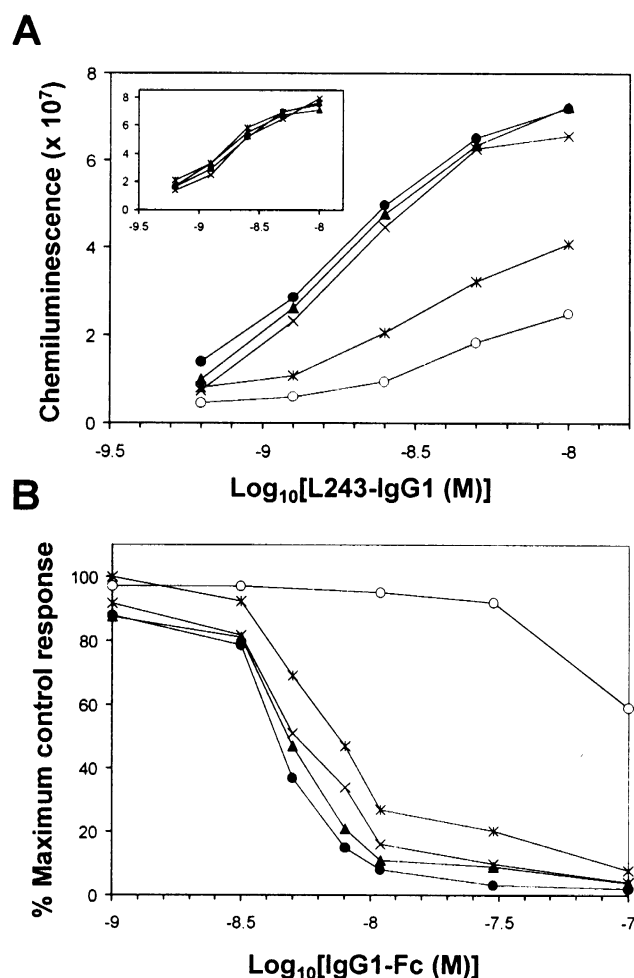


Fig. 4. Superoxide production triggered by homogeneous glycoforms of L243 IgG1 (A), Inhibition of native L243 IgG1 by homogeneous glycoforms of IgG1-Fc Wid (B), ●, native L243 IgG1; ▲, (NGA2F)₂; ×, (M3N2F)₂; *, (M2N2F)₂/(MN2F)₂; ○, deglycosylated L243 IgG1. Inset: results for mock digested L243 IgG1.

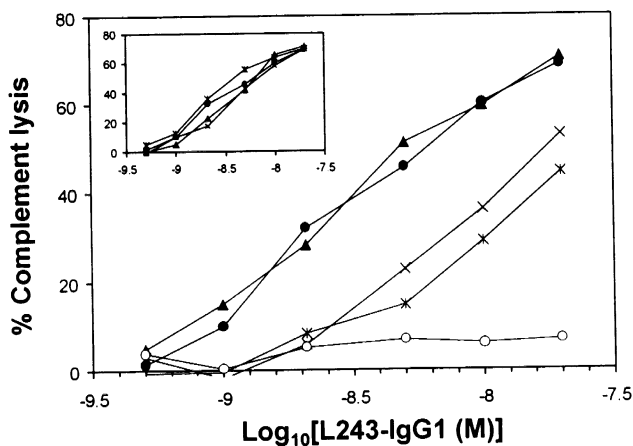


Fig. 5. Complement-mediated lysis activated by native L243 IgG1 and its homogeneous glycoforms. ●, native L243 IgG1; ▲, (NGA2F)2; ×, (M3N2F)2; *, (M2N2F)2/(MN2F)2; ○, deglycosylated L243 IgG1. Inset: results for mock digested L243 IgG1.

that CHO cells can produce human IgG antibodies that express “wild-type” biological activities, however, “mutant” CHO cells can emerge that are limited in the oligosaccharides they synthesise. Thus, Lec-1 cells add high mannose oligosaccharides only and the IgG product is defective in complement activation (Wright and Morrison, 1994). Wright and Morrison (1998) also reported that cells lacking transferases for the addition of sialic acid or galactose yielded IgG with “wild-type” FcγRI, and complement activating abilities, however, an earlier study reported that the fully galactosylated IgG bound FcγRI more avidly than agalactosylated IgG (Tsuchiya et al., 1989). Thus, glycosylation engineering offers the potential to modulate biological activities of IgG antibodies. In the present study we show that truncated oligosaccharides can confer stability and function on IgG-Fc. Our results provide a thermodynamic rationale for the modulation of antibody effector functions through manipulation of glycosylation.

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