

Role of Oligosaccharide Residues of IgG1-Fc in FcγRIIb Binding*

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Engagement of Fcγ receptors (FcγRs) with the Fc region of IgG elicits immune responses by leukocytes. The recent crystal structure of FcγRIII in complex with IgG-Fc has provided details of molecular interactions between these components (Sondermann, P., Huber, R., Oosthuizen, V., and Jacob, U. (2000) *Nature* 406, 267–273). One of the most intriguing issues is that glycosylation of IgG-Fc is essential for the recognition by FcγRs although the carbohydrate moieties are on the periphery of the FcγRIII-Fc interface. To better understand the role of Fc glycosylation in FcγR binding we prepared homogeneous glycoforms of IgG-Fc (Cri) and investigated the interactions with a soluble form of FcγRIIb (sFcγRIIb). A 1:1 complex stoichiometry was observed in solution at 30 °C (K_D , 0.94 μM; ΔG , –8.4 kcal mol^{–1}; ΔH , –6.5 kcal mol^{–1}; $T\Delta S$, 1.9 kcal mol^{–1}; ΔC_p , –160 cal mol^{–1} K^{–1}). Removal of terminal galactose residues did not alter the thermodynamic parameters significantly. Outer-arm GlcNAc residues contributed significantly to thermal stability of the C_H2 domains but only slightly to sFcγRIIb binding. Truncation of 1,3- and 1,6-arm mannose residues generates a linear trisaccharide core structure and resulted in a significantly decreased affinity, a less exothermic ΔH , and a more negative ΔC_p for sFcγRIIb binding, which may result from a conformational change coupled to complex formation. Deglycosylation of the C_H2 domains abrogated sFcγRIIb binding and resulted in the lowest thermal stability accompanied with noncooperative unfolding. These results suggest that truncation of the oligosaccharides of IgG-Fc causes disorder and a closed disposition of the two C_H2 domains, impairing sFcγRIIb binding.

IgG molecules are glycosylated at Asn-297 of the C_H2 domain within the Fc region; the complex biantennary-type oligosaccharides attached have been shown to be essential for Fc effector functions mediated through Fcγ receptors (FcγRs)¹ and

complement C1q (1, 2). Deglycosylation of IgG compromises the recognition by all three cellular FcγRs (3–5). Analysis of carbohydrates isolated from normal human serum IgG has yielded up to 30 different structures. The minimal oligosaccharide structure is a hexasaccharide (GlcNAc₂Man₃GlcNAc) with variable sugar residues attached, which results in the generation of the multiple glycoforms. The oligosaccharide can be traced in x-ray crystal electron density maps of the IgG-Fc and is sequestered within the internal space enclosed by the C_H2 domains. There are extensive noncovalent interactions between the carbohydrate and the protein moiety, resulting in reciprocal influences on conformation (6, 7). Recently we have shown that the C_H2 domains exhibit a progressive reduction in thermal stability and functionality with successive removal of outer-arm sugar residues (8). However, the precise manner in which the oligosaccharide influences or determines Fc structure and functions remains to be determined. Detailed information of the glycosylation influence of IgG-Fc on FcγR binding and complement activation is critical for application of IgG for therapeutic use.

FcγRs are expressed on various hematopoietic cell types (*e.g.* macrophages, eosinophils, neutrophils, natural killer cells, and lymphocytes) and link cellular and humoral immunity by serving as a bridge between antibody specificity and effector cell function. Stimulation of cells through FcγRs results in a wide variety of effector functions, including Ab-dependent cell mediated cytotoxicity (9), phagocytosis (10), oxidative burst (11), and release of inflammatory mediators (12). FcγRs constitute a subfamily of the immunoglobulin gene superfamily and have been classified into three classes (13). FcγRI binds monomeric IgG with high affinity. FcγRII and FcγRIII are low affinity IgG receptors and responsible for the clearance of immune complexes. Furthermore, FcγRs occur in various isoforms (FcγRIa, -b1, -b2, -c; FcγRIIa1–2, -b1–3, -c) and alleles (FcγRIIa1-HR, -LR; FcγRIIb-NA1, NA2) (14). Indirect protein engineering approaches suggest that interaction sites for the cellular FcγRs are localized to the hinge proximal region of the C_H2 domain (4, 15–17). Crystal structures have been reported for extracellular portions of FcγRIIa (18) and FcγRIIb (19), suggesting conflicting models for the formation of IgG-Fc-FcγR complexes. Direct evidence has now been provided from the crystal complex of human IgG1-Fc with recombinant human FcγRIII (20), and NMR studies of the interaction of mouse IgG2b-Fc with mouse FcγRII (21), demonstrating a 1:1 stoichiometry as the consistent requirement for the expression of Fc effector functions without triggering permanent activation through a single IgG molecule.

linked pentasaccharide cores; (MN2F)₂, IgG-Fc glycoform with fucosylated N-linked trisaccharide cores.

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¹ The abbreviations used are: FcγR, receptor for Fc portion of IgG; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; (NGA2F)₂, biantennary nongalactosylated and fucosylated IgG-Fc glycoform; (M3N2F)₂, IgG-Fc glycoform with fucosylated N-

To better understand how the oligosaccharide contributes to the expression of the IgG-Fc effector function, we investigated the interaction between a recombinant soluble form of Fc γ RIIb (sFc γ RIIb) and homogeneous Fc glycoforms using isothermal titration calorimetry and surface plasmon resonance. A 1:1 stoichiometry for the interaction of sFc γ RIIb with Fc in solution is demonstrated although the binding of two sFc γ RIIb molecules to one IgG molecule has been proposed by a modeling approach (19). We extend this finding to examine the thermodynamic analysis of defined glycoforms of IgG-Fc on sFc γ RIIb binding and show that the mannose residues exert an important influence on sFc γ RIIb binding. Finally, we discuss how the oligosaccharide residues of IgG-Fc affect Fc γ R binding.

EXPERIMENTAL PROCEDURES

Proteins

sFc γ RIIb—Recombinant soluble human Fc γ RIIb was expressed in *Escherichia coli*, refolded, and purified, as described previously (22). Briefly, the extracellular region of hFc γ RIIb2 (23) was overexpressed as inclusion bodies in *E. coli* which were purified by lysozyme treatment of the cells and subsequent sonication. The resulting suspension was centrifuged (30 min, 30,000 $\times g$) and washed with buffer containing 0.5% (w/v) lauryldimethylamine oxide. The centrifugation step and resuspension in lauryldimethylamine oxide-containing buffer was repeated twice, and then twice more without lauryldimethylamine oxide. The inclusion bodies were dissolved in 6 M guanidine hydrochloride and the protein renatured. The dialyzed and filtered protein solution was applied to a human IgG-Sepharose column and eluted by pH jump. The concentrated neutralized fractions were subjected to size-exclusion chromatography on a Superdex-75 column (Amersham Pharmacia Biotech). Protein concentrations were determined spectrophotometrically using a calculated ϵ_{280} of 29,400 M⁻¹ cm⁻¹ (24).

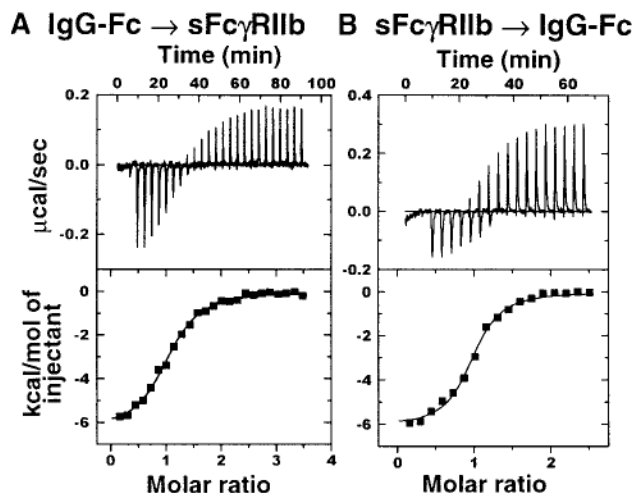


FIG. 1. Stoichiometry of the interaction between IgG-Fc and sFc γ RIIb. A, Fc-into-sFc γ RIIb titration. Five μ l of Fc (0.37 mM) was injected into the cell containing 10 μ M sFc γ RIIb at 30 $^{\circ}$ C, except for the first injection which was 1 μ l. B, sFc γ RIIb-into-Fc titration. Eight μ l of sFc γ RIIb (0.33 mM) was injected into the cell containing 14 μ M Fc except for the first injection which was 1 μ l. Top, raw heat data obtained for a series of injections. Bottom, integrated curve showing the best fit. The data were fitted to a "one-set of sites" model. The data set presented is one of two sets of independent experiments.

TABLE I

Thermodynamics of the interaction between IgG (Cri) and sFc γ RIIb by ITC at 30 $^{\circ}$ C

The data are the mean values of the two independent experiments with an S.E.

Titration	<i>n</i> (Stoichiometry)	<i>K_d</i>	ΔG	ΔH	<i>T</i> ΔS
		μ M		kcal/mol	
IgG1-Fc $\rightarrow$ sFc γ RIIb	1.00 $\pm$ 0.04	0.98 $\pm$ 0.01	-8.30 $\pm$ 0.01	-6.20 $\pm$ 0.20	2.10 $\pm$ 0.20
sFc γ RIIb $\rightarrow$ IgG1-Fc	0.91 $\pm$ 0.01	0.94 $\pm$ 0.06	-8.35 $\pm$ 0.04	-6.49 $\pm$ 0.27	1.86 $\pm$ 0.31
sFc γ RIIb $\rightarrow$ intact IgG1	0.97 $\pm$ 0.04	0.95 $\pm$ 0.02	-8.35 $\pm$ 0.01	-6.35 $\pm$ 0.27	2.00 $\pm$ 0.28
sFc γ RIIb $\rightarrow$ R/A IgG1 ^a	1.06 $\pm$ 0.02	1.89 $\pm$ 0.40	-7.93 $\pm$ 0.13	-3.80 $\pm$ 0.06	4.12 $\pm$ 0.13

^a Reduced and alkylated IgG.

Homogeneous Glycoforms of IgG-Fc (Cri)—The monoclonal protein IgG1 (Cri, allotype G1m(f)) was isolated from serum from a patient with multiple myeloma and the IgG1-Fc fragment generated as previously reported (25). The following protocols were used to generate truncated glycoforms of IgG1-Fc (Cri). For (NGA2F)₂ glycoform, the native IgG1-Fc (5 mg) in 50 mM acetate buffer, pH 5.0, was exposed to sialidase (0.5 unit; *Arthrobacter ureafaciens*, Roche Molecular Diagnostics, East Sussex, UK) at 37 $^{\circ}$ C for 24 h. The pH was adjusted to 6.0 and β -galactosidase (0.2 unit; *Diplococcus pneumoniae*, Roche Molecular Biochemicals) added and the solution incubated at 37 $^{\circ}$ C for 24 h. For the (M3N2F)₂ glycoform, (NGA2F)₂ IgG1-Fc (5 mg), in 50 mM citric acid, Na₂HPO₄, pH 4.8, was exposed to *N*-acetyl- β -D-glucosaminidase (0.225 unit, *D. pneumoniae*, Roche Molecular Biochemicals) at 37 $^{\circ}$ C for 24 h. The (MN2F)₂ glycoform, (M3N2F)₂ IgG1-Fc (5 mg) in 50 mM acetate buffer containing zinc acetate (2 mM), pH 5.0, was exposed to α -mannosidase (15 units, Jack bean, Glyko, Oxfordshire, UK) at 37 $^{\circ}$ C for 48 h. For deglycosylated IgG1-Fc the native IgG1-Fc (5 mg) in 40 mM KH₂PO₄, 10 mM EDTA, pH 7.4, was exposed to peptide-*N*-glycosidase F (30 units; *Flavobacterium meningosepticum*, Roche Molecular Biochemicals) at 37 $^{\circ}$ C for 72 h. Following exposure to glycosidases all proteins were affinity purified using a Streptococcal protein G-Sepharose 4B (Amersham Pharmacia Biotech) column, eluted with 0.1 M glycine-HCl buffer, pH 2.7. Eluates were immediately neutralized by the addition of 1 M Tris/HCl, pH 9.0, and dialyzed extensively against phosphate-buffered saline.

Cleavage of Interchain Disulfide Bridges

The IgG1 or IgG1-Fc (Cri) was reduced and alkylated according to Fleischman *et al.* (26). Briefly, IgG1 or IgG1-Fc (Cri, 10 mg/ml) was dissolved in 0.5 M Tris/HCl containing 2 mM EDTA, pH 8.0. The protein was treated with 0.01 M dithiothreitol at room temperature for 1 h after being sparged with N₂ gas. Iodoacetamide was added to a final concentration of 0.02 M and incubated on ice for 30 min with gentle stirring. The protein was dialyzed extensively against phosphate-buffered saline. Cleavage of the interchain disulfide bonds was confirmed by nonreduced SDS-polyacrylamide gel electrophoresis and electrospray ionization mass spectrometry. Alkylation of Cys residues involved in inter-chain disulfide bridges (L chain, 1; H chain, 3) was confirmed by an increase in their masses by 59 and 178 Da, respectively, relative to those of reduced L and H chain (data not shown), which corresponds well to the mass of the -CH₂-CONH₂ group (58 Da) added by alkylation.

Electrospray Ionization Mass Spectrometry

Electrospray ionization mass spectrometry was performed using an LCTTM instrument and MassLynx data acquisition (Micromass, Manchester, UK). 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 Cri proteins (1 mg/ml) were extensively dialyzed against distilled water and formic acid added to a final concentration of 1% (v/v). Ten microliters of the sample solution was injected via a Rheodyne valve into the mobile phase of 50% acetonitrile, and 50% of 0.1% (v/v) formic acid in water. Ten to twenty spectra were averaged, baseline subtracted, and deconvoluted using MAX.ENT. software (Micromass).

Isothermal Titration Calorimetry (ITC)

ITC was performed at 30.0 $^{\circ}$ C using the MCS-ITC system (MicroCal, Northampton, MA). To examine the stoichiometry of the interaction between IgG-Fc and sFc γ RIIb, one of the proteins was dissolved in phosphate-buffered saline, pH 7.4, to 10–13 μ M and placed in the calorimeter cell (1.34 ml). The injection syringe (250 μ l) was filled with a concentrated solution (0.30–0.37 mM) of the other component dissolved in the same buffer. sFc γ RIIb from the syringe was titrated into

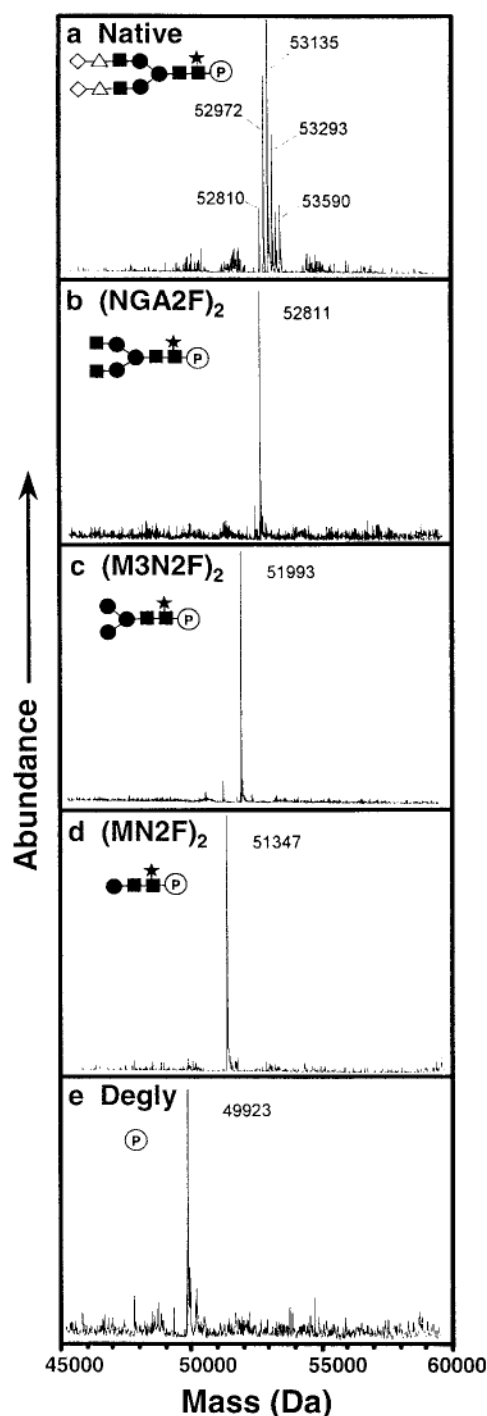


FIG. 2. Electrospray ionization mass spectrometry of homogeneous glycoforms of IgG1-Fc (Cri). a, native Fc; b, Fc treated with sialidase and β -galactosidase; c, Fc treated with sialidase, β -galactosidase, and *N*-acetylglucosaminidase; d, Fc treated with sialidase, β -galactosidase, *N*-acetylglucosaminidase, and α -mannosidase; e, peptide-*N*-glycosidase F-treated protein. Degly refers to deglycosylated protein. Open symbols in panel a represent variable attachment of terminal galactose and sialic acid residues; The sugars are represented as follows: ★, fucose; ■, *N*-acetylglucosamine; ●, mannose; △, galactose; ◇, sialic acid; p, protein.

the calorimeter cell containing the homogeneous glycoform of IgG-Fc. A series of injections was made, and the observed heat pulses integrated and fitted to a theoretical titration curve using software Origin 2.9 (MicroCal) with ΔH (the enthalpy change in cal mol⁻¹), K_d (the equilibrium association constant in M⁻¹), and n (the number of binding sites per monomer) as adjustable parameters. The quantity c values (27) were between 5 and 20 in the present study.

TABLE II
Determination of the glycoforms of IgG1-Fc (Cri) by Electrospray ionization mass spectrometry

Glycoform	Molecular mass (Da)	
	Observed	Calculated
Deglycosylated	49,923	49,931
(MN2F) ₂	51,347	51,358
(M3N2F) ₂	51,993	52,007
(NGA2F) ₂	52,811	52,820
NGA2F + NA1F ^a	52,972	52,982
NGA2F + NA2F/(NA1F) ₂ ^b	53,135	53,144
NA1F + NA2F	53,293	53,306
Monosialyl NA1F + NA2F	53,590	53,598

^a NA1F, fucosylated and monogalactosylated glycoform.

^b NA2F, fucosylated and digalactosylated glycoform.

The thermodynamic parameters were calculated from basic equations of thermodynamics: $\Delta G = \Delta H - T\Delta S = -RT \ln K_d$, where ΔG , ΔH , and ΔS are the changes in free energy, enthalpy, and entropy of binding, respectively. T is the temperature in K, and $R = 1.98$ cal mol⁻¹ K⁻¹.

The change in heat capacity of binding (ΔC_p), which is assumed to be independent of temperature, was determined using the following expression: $\Delta C_p = (\Delta H_{T_2} - \Delta H_{T_1})/(T_2 - T_1)$.

Differential Scanning Microcalorimetry (DSC)

Experiments were performed on a VP-DSC microcalorimeter (MicroCal) 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) was observed at this low concentration. Data were collected and analyzed using Origin 4.1 software (MicroCal), essentially as previously described (28). The area below each transition peak corresponds to ΔH , and the van't Hoff enthalpy (ΔH_v) was calculated using the formula: $\Delta H_v = 4RT_m^2 <\Delta C_p>_{tr,max}/\Delta H$, where $<\Delta C_p>_{tr,max}$ is the measured heat capacity at the peak maximum, and R is the gas constant. The cooperativity of the heat transition was calculated as the ratio $\Delta H/\Delta H_v$ (29).

Surface Plasmon Resonance Measurements

sFcγRIIb binding was measured using BIAcore 3000 and CM5 biosensor chips (BIAcore, Uppsala, Sweden). Homogeneous glycoforms of IgG-Fc were immobilized onto the chips by amide coupling following the manufacturer's instructions. Assays were performed with sFcγRIIb in a mobile phase at flow rate of 10 μ l/min using 20 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.05% (v/v) surfactant P20, pH 7.4. The carbodiimide/ethanolamine-treated blank was included as a control. K_d values for the binding of the Fc glycoforms to sFcγRIIb were estimated from the plots of the steady-state binding levels against sFcγRIIb concentrations fitted to a single binding site model using the formula: $R_{eq} = R_{max} * c/(K_d + c)$, where R_{eq} is the level of binding at equilibrium, and R_{max} is the theoretical binding capacity, and c is the concentration of sFcγRIIb. Curve fitting was performed with Origin 5.0 (MicroCal). Experimental ΔG_T values were fitted using Origin 5.0 to nonlinear van't Hoff equation with a reference temperature T_o of 303.15 K to obtain ΔH_{T_o} , ΔS_{T_o} , ΔC_p , assuming that the change in heat capacity for the association, ΔC_p is a temperature-independent variable (30): $\Delta G_T = \Delta H_{T_o} - T\Delta S_{T_o} + (T - T_o)\Delta C_p - T\Delta C_p \ln(T/T_o)$.

RESULTS

Stoichiometry of the Interaction between IgG1-Fc and sFcγRIIb in Solution—To determine the independent thermodynamic parameters (K_d and ΔH) as well as the number of binding sites (n) governing IgG-sFcγRIIb interaction, ITC was carried out at 30 °C (Fig. 1). The thermodynamic characteristics for this interaction was enthalpy- and entropy-driven (Table I), which is markedly different from IgG-sFcγRIII interaction where a large unfavorable entropy is compensated by a larger favorable enthalpy at 30 °C (31, 32). Essentially the same thermodynamic parameters were obtained irrespective of whether IgG-Fc was titrated into sFcγRIIb (Fig. 1A) or sFcγRIIb into IgG1-Fc (Fig. 1B) (Table I). Both binding isotherms were best fitted to a 1:1 association model. If the stoichiometry were different from 1:1, the values of ΔH and n would differ depending on which component is in the calorimeter cell and which is being titrated as shown for the interac-

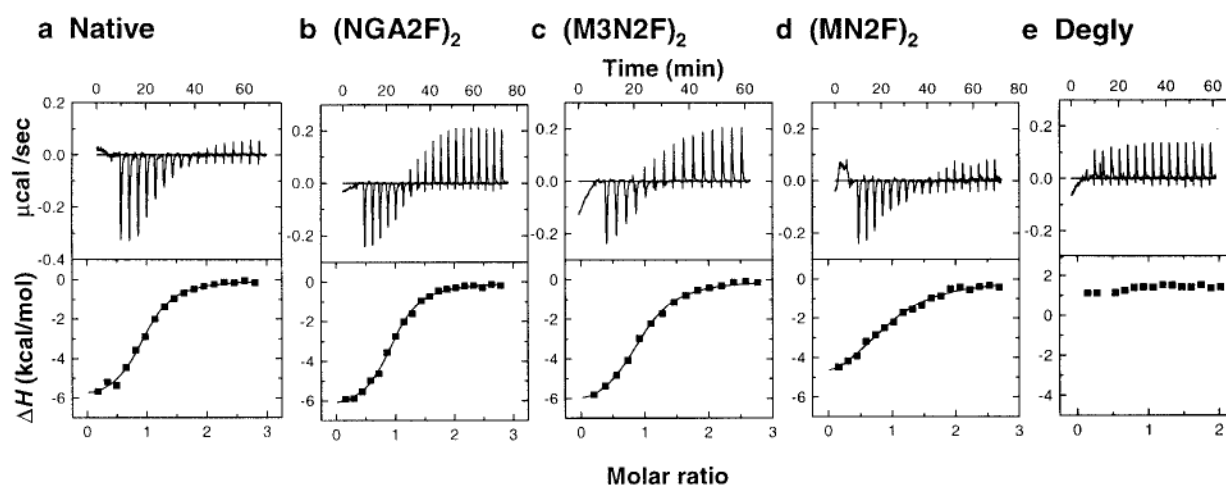


FIG. 3. Binding isotherms for the interactions between sFc γ RIIb and homogeneous glycoforms of IgG1-Fc (Cri). Eight μ l of sFc γ RIIb (0.30 mM) was injected into the cell containing a homogeneous IgG1-Fc glycoform (13 μ M) at 30 $^{\circ}$ C except the first injection that was 1 μ l. The data were fitted to a one-set of sites model. The data set presented is one of two similar experiments.

TABLE III

Thermodynamic parameters for the interactions between sFc γ RIIb and homogeneous glycoforms of IgG1-Fc by ITC at 30 $^{\circ}$ C

The parameters were calculated from the binding isotherms in Fig. 3. K_d is given in units of μ M, ΔG , ΔH , and $T\Delta S$ are given in kcal/mol, and ΔC_p is given in cal/mol/K. sFc γ RIIb was injected into the calorimeter cell containing various IgG-Fc glycoforms. The parameters except ΔC_p are the mean values of the two independent experiments with an S.E.

Glycoform	Stoichiometry	K_d	ΔG	ΔH	$T\Delta S$	ΔC_p
Native	0.91 ± 0.01	0.94 ± 0.06	-8.35 ± 0.04	-6.49 ± 0.27	1.86 ± 0.31	-160 ± 10^a
(NGA2F) $_2$	0.93 ± 0.01	0.99 ± 0.01	-8.33 ± 0.01	-6.47 ± 0.06	1.85 ± 0.06	ND ^b
(M3N2F) $_2$	0.96 ± 0.07	1.19 ± 0.04	-8.21 ± 0.02	-6.58 ± 0.21	1.63 ± 0.14	ND ^b
(MN2F) $_2$	0.90 ± 0.02	3.03 ± 0.55	-7.63 ± 0.12	-5.89 ± 0.15	1.74 ± 0.27	-250 ± 40^a

^a The errors represent the estimated standard error obtained from the data fitting.

^b ND, not determined.

tion between IgG and neonatal FcR (33). Thus, the results verify that the stoichiometry for the interaction is 1:1 in solution, suggesting that sFc γ RIIb binds to the hinge-proximal region of the C_H2 domains of Fc in a manner similar to that observed for the structure of Fc in complex with sFc γ RIII (20). The thermodynamic parameters for sFc γ RIIb binding to intact IgG molecules were almost the same as those for IgG-Fc (Table I), indicating that the Fab regions of IgG do not contribute significantly to binding of Fc γ RIIb. We disrupted the hinge inter-chain disulfide bridges of IgG by mild reduction and alkylation to examine its effect on the stoichiometry of the interaction. The stoichiometry for sFc γ RIIb interaction with reduced and alkylated IgG remained the same as that for sFc γ RIIb-intact IgG interaction (1:1) although the affinity was reduced by a factor of two (Table I). Thus the stoichiometry of binding is unaffected by the integrity of the hinge disulfide bridges of IgG.

Electrospray Ionization Mass Spectrometry of Homogeneous Glycoforms of IgG-Fc—To dissect the roles of Fc oligosaccharide residues in sFc γ RIIb binding, exoglycosidases were used to prepare Fc glycoforms bearing truncated oligosaccharides. The purity and glycosylation homogeneity of the glycoforms of IgG1-Fc (Cri) were tested by electrospray ionization mass spectrometry (Fig. 2). The observed mass of deglycosylated Fc (49,923 Da) was in good agreement with the theoretical mass of the Fc fragment (49,931 Da; calculated for the G1m(f) allotype) lacking the C-terminal lysine, and having Asp at Asn-297. The mass spectra of native Fc showed heterogeneity in terms of glycosylation as reported by Jefferis *et al.* (25). Experimental masses of the truncated glycoforms were in good agreement with the calculated mass values within an error of 0.03% (Table II).

Influence of Glycoform of IgG1-Fc on sFc γ RIIb Binding—The

interactions between sFc γ RIIb and homogeneous glycoforms of IgG-Fc (Cri) were investigated by ITC (Fig. 3). The thermodynamic parameters were characterized at 30 $^{\circ}$ C (Table III). The native Fc and its nongalactosylated glycoform, (NGA2F) $_2$, showed very similar thermodynamic parameters (Fig. 3, *a* and *b*; Table III). Removal of terminal GlcNAc residues to generate the (M3N2F) $_2$ glycoform resulted in only a slight decrease in binding affinity without a significant change in enthalpy, indicating a less favorable entropic contribution (Fig. 3*c*, Table III). However, trimming of the terminal mannose residues generated the (MN2F) $_2$ glycoform that showed a marked reduction in binding affinity and enthalpy without a significant change in entropy (Fig. 3*d*, Table III). Interaction of deglycosylated Fc with sFc γ RIIb was not detectable (Fig. 3*e*). Mock-digested Fc showed essentially the same thermodynamics for sFc γ RIIb binding as the native Fc (data not shown).

Surface plasmon resonance was employed for thermodynamic analysis of the interactions of sFc γ RIIb with homogeneous glycoforms of Fc immobilized on sensor chips at six different temperatures 5, 10, 15, 20, 25, and 30 $^{\circ}$ C. A representative binding profile is shown at 25 $^{\circ}$ C in Fig. 4*A*. Although the dissociation constants obtained by surface plasmon resonance were generally greater by about 3-fold (Table IV), relative to those obtained by ITC (Table III), both data sets show the same trends in that trimming of terminal GlcNAc and mannose residues of the oligosaccharide chains of IgG-Fc results in a slight and marked decrease in affinity, respectively (Fig. 4*B*). In addition, it was shown that the binding affinity to sFc γ RIIb was decreased with increasing the temperature for all glycoforms (Fig. 4*B*). The plots of ΔG values against temperature were fitted to a nonlinear van't Hoff equation (Fig. 4*C*). Apparently, the thermodynamics were almost the same between native Fc and the (NGA2F) $_2$ glycoform because their plots over-

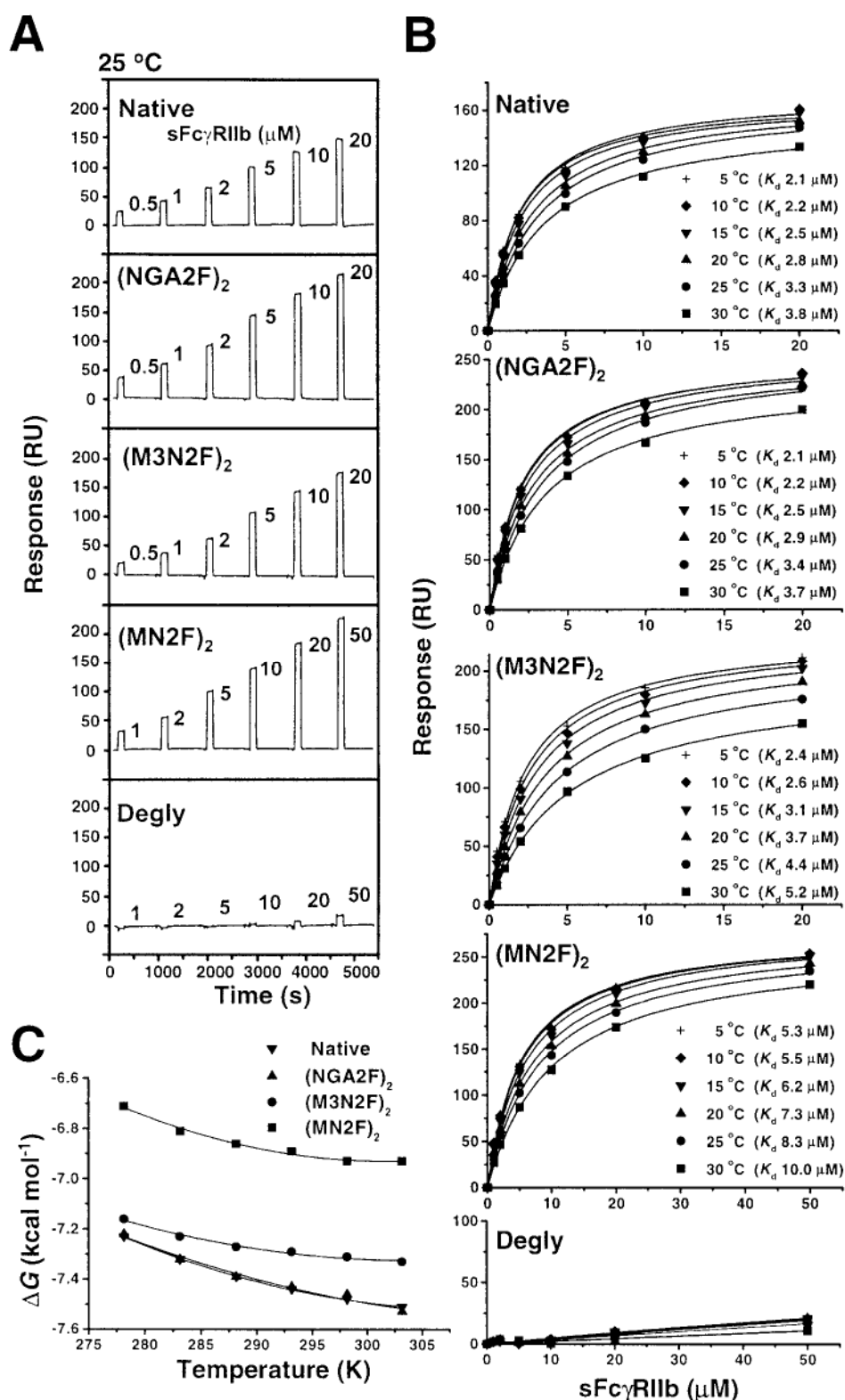


FIG. 4. BIAcore analysis of the interactions between sFc γ RIIb and homogeneous Fc glycoforms. *A*, sFc γ RIIb was injected at six concentrations indicated in each panel at a flow rate of 10 μ L/min on a biosensor CM5 chip with channels coupled with homogeneous glycoforms of IgG1-Fc. Binding sensorgrams were produced by subtraction of the signal from a carbodiimide/ethanolamine-treated blank channel. Two independent experiments were carried out at six temperatures from 5 to 30 °C. An experiment at 25 °C is shown as a representative. *B*, plots of mid-injection peak data against sFc γ RIIb concentrations. The data were fitted to a single binding site model for receptor binding to homogeneous glycoforms of IgG-Fc. K_d values shown are the average of two independent experiments. *C*, temperature dependence of ΔG of sFc γ RIIb binding to each IgG-Fc glycoform. The plots were fitted to a nonlinear van't Hoff equation (see "Experimental Procedures").

lapped closely (Fig. 4C). Interestingly, ΔC_p of the (M3N2F)₂ glycoform was similar to those of native Fc and the (NGA2F)₂ glycoform, however, the value for the (MN2F)₂ glycoform was more negative (Table IV).

Thermal Stability of the Homogeneous Glycoforms of IgG1-Fc (Cri)—The thermal stability of homogeneous glycoforms of IgG1-Fc (Cri) was examined by DSC (Fig. 5). The first transition peak has been assigned to the unfolding of the C_H2 do-

TABLE IV
Thermodynamic parameters determined by surface plasmon resonance at 30 °C

The parameters were calculated from the data in Fig. 4, B and C. Units for K_d , ΔG , ΔH , $T\Delta S$, and ΔC_p are as in Table III. T_s is given in K and ΔS_{HE} (T_s), ΔS_{rt} , and excess entropy are given in cal/mol/K. The errors represent the estimated standard error obtained from the data fitting.

Glycoform	K_d	ΔG	ΔH	$T\Delta S$	ΔC_p	T_s^a	$\Delta S_{HE}(T_s)^b$	ΔS_{rt}^c	Excess entropy
Native	3.8 ± 0.2	-7.51 ± 0.04	-6.40 ± 0.16	1.10 ± 0.17	-170 ± 10	309	51	-50	1
(NGA2F) ₂	3.7 ± 0.3	-7.53 ± 0.03	-5.70 ± 0.92	1.81 ± 0.93	-130 ± 70	317	35	-50	0
(M3N2F) ₂	5.2 ± 0.2	-7.33 ± 0.02	-7.16 ± 0.45	0.16 ± 0.46	-130 ± 30	303	42	-50	0
(MN2F) ₂	10.0 ± 0.5	-6.93 ± 0.03	-7.23 ± 0.55	-0.30 ± 0.55	-220 ± 40	303	72	-50	22

^a The temperature at which entropy contribution becomes zero. $\Delta C_p = \Delta S_f / \ln(T/T_s)$; $T = 303.15$ K.

^b Entropy change from the hydrophobic effect at T_s : $\Delta S_{HE}(T_s) = 1.35\Delta C_p \ln(T_s/386)$ (Ref. 41).

^c Unfavorable entropy contribution due to the loss of rotational and translational degrees of freedom.

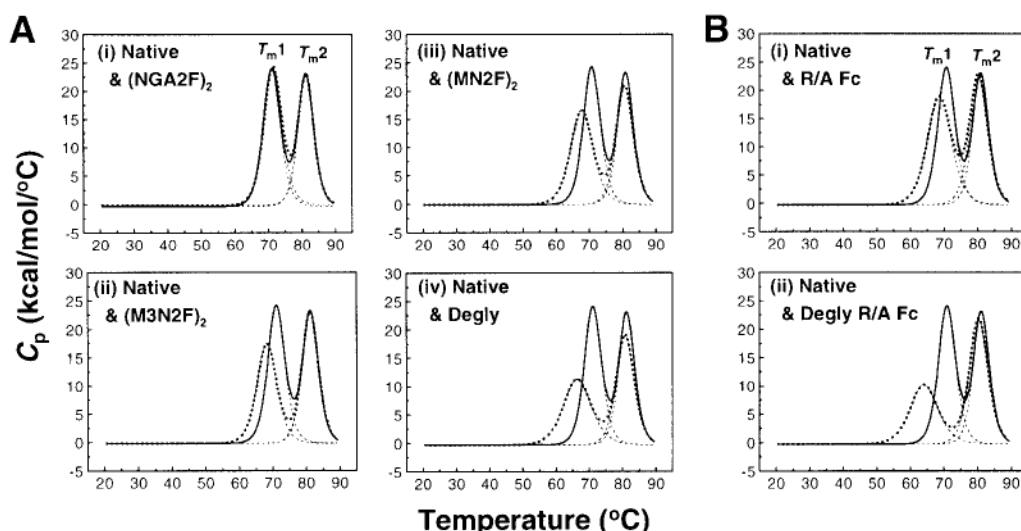


FIG. 5. Deconvoluted thermograms of homogenous glycoforms of IgG-Fc (Cri). A, native, (NGA2F)₂, (M3N2F)₂, (MN2F)₂, and deglycosylated Fc (Cri). B, reduced and alkylated native and deglycosylated Fc (Cri). In each thermogram the solid line represents the profile for native IgG-Fc. The data were baseline corrected. The thermograms presented are one of two similar experiments.

main and the second to the C_H3 domains (34). The T_m 1 of IgG-Fc (Cri) (Fig. 5A) exhibited a similar dependence to IgG1-Fc (Wid) on progressive removal of the oligosaccharide moiety (8). Removal of the terminal GlcNAc residues resulted in a marked decrease in T_m 1 by 3.1 °C and enthalpy of unfolding (Fig. 5A, ii; Table V), indicating destabilization of the C_H2 domains. The T_m 1 of the (MN2F)₂ glycoform did not alter significantly following trimming of the mannose residues (Fig. 5A, iii; Table V) although binding affinity of this glycoform to sFcγRIIb was reduced 3-fold, relative to that of (M3N2F)₂ glycoform (Fig. 3, Table III). Deglycosylated Fc showed further reductions in T_m 1 and enthalpy (Fig. 5A, iv; Table V).

All glycoforms except deglycosylated Fc exhibited a ratio of calorimetric enthalpy (ΔH) to van't Hoff enthalpy (ΔH_v) that was close to unity (Table V). This indicates that the two C_H2 domains unfold cooperatively as a single unit, highlighting the possibility that the two oligosaccharides make contacts with each other. In contrast, reduced and alkylated Fc lacking the hinge disulfide bridges showed a ratio close to two (Fig. 5B, i; Table V). This is consistent with the presence of two cooperative units per molecule: the now mobile C_H2 domains unfold independently of each other. Interestingly, removal of the oligosaccharide chains from native and reduced and alkylated Fc resulted in noncooperative melting of the C_H2 domains, such that $\Delta H_1/\Delta H_v$ was 1.5 for deglycosylated Fc and 1.4 for deglycosylated and reduced and alkylated Fc, suggesting that both samples unfold similarly, *i.e.* that there is at least one intermediate between the folded and unfolded states (Fig. 5, A, iv, and B, ii).

DISCUSSION

Recent crystal structures of the sFcγRIII-Fc complex have revealed the detailed molecular interactions between these components (20, 35). However, while in one structure a primary GlcNAc residue participates directly in binding to the receptor, in the other there is no direct interaction of the oligosaccharide with the receptor. Thus, it is unclear as to why the oligosaccharide at Asn-297 of IgG-Fc on the periphery of the receptor interaction site is critical for FcγR binding. We attempted to probe the roles of the N-linked oligosaccharide chains of IgG-Fc in binding to sFcγRIIb by thermodynamic analysis of the interactions between sFcγRIIb and IgG-Fc bearing truncated oligosaccharides. We found that removal of the mannose residues on the 1,6- and 1,3-arms of the oligosaccharide of IgG-Fc has a significant influence on the receptor binding. The cause of the inability of deglycosylated IgG to bind FcγR is also discussed.

Stoichiometry—We established a 1:1 stoichiometry for the interaction between sFcγRIIb and IgG-Fc in solution. The molecular basis for the 1:1 stoichiometry has been detailed for the sFcγRIII-IgG-Fc complex in the crystalline state (20). sFcγRIII molecules bind asymmetrically at a 1:1 ratio to the lower hinge region of both C_H2 domains, a primary GlcNAc sugar residue and residues in the vicinity of the carbohydrate. The decreased binding of reduced and alkylated IgG to sFcγRIIb provides an insight into the role of inter-chain disulfide bridges in receptor binding (Table I). This result supports the involvement of both C_H2 domains of IgG in sFcγRIIb binding, suggesting that the disulfide bridges are not essential for a 1:1 association but contribute to appropriate positioning of both C_H2 domains to

TABLE V
Thermodynamic parameters for the unfolding of homogenous glycoforms of human IgG1-Fc (Cri) by DSC

The parameters were calculated from the thermograms in Fig. 5. The data are the mean values of the two independent experiments with an S.E.

Glycoform	1 st transition				2 nd transition			
	T_m1	$\Delta H1$	ΔH_v1	$\Delta H1/\Delta H_v1$	T_m2	$\Delta H2$	ΔH_v2	$\Delta H2/\Delta H_v2$
	°C	kcal/mol			°C	kcal/mol		
Native	71.0 ± 0.02	151 ± 4	141 ± 5	1.1	81.3 ± 0.05	142 ± 3	153 ± 3	0.9
(NGA2F) ₂	71.2 ± 0.04	152 ± 8	135 ± 1	1.1	82.4 ± 0.03	120 ± 3	166 ± 2	0.7
(M3N2F) ₂	67.9 ± 0.04	125 ± 16	115 ± 1	1.1	81.4 ± 0.04	125 ± 8	160 ± 5	0.8
(MN2F) ₂	67.7 ± 0.08	131 ± 1	105 ± 1	1.2	81.2 ± 0.06	129 ± 4	146 ± 4	0.9
Deglycosylated	65.7 ± 0.16	120 ± 7	82 ± 4	1.5	81.0 ± 0.02	120 ± 3	141 ± 1	0.9
R/A ^a	68.4 ± 0.04	167 ± 5	88 ± 1	1.9	79.6 ± 0.03	147 ± 7	143 ± 2	1.0
Deglycosylated R/A	63.9 ± 0.02	108 ± 1	80 ± 1	1.4	79.4 ± 0.02	114 ± 2	124 ± 2	0.9

^a Reduced and alkylated Fc.

optimize FcγR binding. Using truncated Fc mutants we recently showed that hinge disulfide bonds are not necessarily required for recognition by FcγRI of IgG1 or single-chain Fv fusion protein with Fc (36). However, the mutants lacking C_H3 domains (IgG lacking a C_H3 domain pair; single-chain Fv fusion protein with a hinge-C_H2 domain) fail to be recognized by FcγRI following mild reduction and alkylation. Therefore it seems likely that the C_H3-C_H3 and C_H2-C_H3 contacts, 1090 Å² and 389 Å², respectively (6), maintain the close apposition of the two C_H2 domains even in the absence of hinge disulfide bonds. Taken together, two C_H2 domains are required for recognition of IgG by FcγRs, either paired covalently at the N-terminal end by hinge disulfide bonds or noncovalently at the C-terminal end by the C_H3 domain pair. Interestingly, the oligosaccharide-C_H2 contact (522 Å²) is an additional requirement for optimal FcγR binding as shown in Fig. 3e. The contribution of individual sugar residues of the oligosaccharides to sFcγRIIb binding is discussed below.

Significance of Galactose Residues—We obtained no evidence for the positive contribution of terminal galactose residues to sFcγRIIb binding or the thermal stability of the C_H2 domains (Figs. 3b, 4, and 5A, i; Tables III, IV, and V). The report that in the absence of the 1,6-arm galactose residue the oligosaccharides of IgG-Fc becomes freely mobile (37) does not correspond to our findings because we could not detect deglycosylated IgG-like behavior either in the binding isotherm (Fig. 3b) or thermogram (Fig. 5A, i). These results are consistent with our previous mutational studies, which showed that replacement of Lys-246, Asp-249, and Glu-258 that make contacts with 1,6-arm galactose does not affect the recognition of IgG by FcγRII (38). It is logical to conclude therefore, that even in the absence of galactose residues, the equilibrium for the oligosaccharide-C_H2 domain interaction inclines toward association. On the other hand, it has been reported that terminal GlcNAc residues of agalactosyl IgG-Fc can be recognized by the mannose binding lectin (39) and the mannose receptor on macrophages and dendritic cells (40). These findings may be relevant to the pathogenesis of rheumatoid arthritis since it has been shown that serum IgG is hypogalactosylated in this disease (39).

Significance of GlcNAc Residues—Removal of the terminal GlcNAc residues results in a significant decrease in the thermal stability of the C_H2 domains (Fig. 5A, ii; and Table V) but in only a minor decrease of affinity for sFcγRIIb (Fig. 3c; Table III). This result supports our previous work on site-directed mutagenesis in which Asp-249 and Lys-246, making contacts with the terminal galactose and GlcNAc residues, are replaced with Ala and showed minimal influences on FcγRI and FcγRII binding (38). Therefore, it seems likely that loss of the GlcNAc-C_H2 domain interactions cause local disorder in the area of the C_H2 domain distant from the lower hinge and hence does not

markedly influence binding of the receptor. The minor decrease in affinity of the (M3N2F)₂ glycoform for sFcγRIIb results solely from changes in the entropic contribution (Table III). Such complex formation involves the intramolecular conversion of a partially unstructured C_H2 polypeptide chain into a more ordered structure. This reduction in the conformational entropy is more pronounced for the (M3N2F)₂ glycoform, reflecting a more disordered free state.

Significance of Mannose Residues—A marked reduction in binding affinity of the (MN2F)₂ glycoform for sFcγRIIb (Figs. 3d and 4; Tables III and IV) demonstrated that the 1,3- and 1,6-arm mannose residues are among the key sugar residues which influence sFcγRIIb binding. This finding is in good agreement with our recent results obtained by superoxide production assay in which recognition of the (MN2F)₂ glycoform of L243-IgG1 by FcγRI is significantly impaired, relative to the native IgG glycoform (8). As deglycosylated Fc did not show measurable affinity for sFcγRIIb by ITC (Fig. 3e), the (MN2F)₂ glycoform, which shows intermediate affinity, may provide insight into the glycoform requirements of IgG-Fc for FcγR binding. The 1,6-arm mannose residue makes contacts with Phe-241 and Phe-243 of the C_H2 domains (7). On the other hand, the 1,3-arm mannose makes contacts with the counterpart mannose residue of the other oligosaccharide chain, which may support a minimum separation between the C_H2 domains (20). Although it seems likely that removal of the 1,3-arm mannose residues abolishes the contact between the two C_H2 domains, the DSC results strongly suggest that C_H2-C_H2 contact is maintained without the mannose residues because the ratio $\Delta H1/\Delta H_v1$ of the C_H2 domains for (MN2F)₂ was similar to the value for the native protein (Table V). If the C_H2 domains of (MN2F)₂ did not contact each other and hence were unfolded independently by the temperature rise, then the ratio $\Delta H1/\Delta H_v1$ would be close to 2 as observed for reduced and alkylated Fc. Therefore, it is very likely that removal of the mannose residues results in a decrease in the distance between the two lower hinge regions because the oligosaccharides are no longer biantennary structures. According to the crystal structure of the sFcγRIIb-IgG-Fc complex (20), the horseshoe-shaped Fc opens up on complex formation, with the distance between the Pro-329 residues located on the N-terminal tip of both C_H2 domains increased by 7 Å. Taken together, the decrease in the distance between the two C_H2 domains of uncomplexed Fc because of removal of the mannose residues may have a negative influence on sFcγRIIb binding.

ΔH and ΔC_p for the interaction between the (MN2F)₂ glycoform and sFcγRIIb (Tables III and IV) suggest a conformational change coupled to complex formation due to the folding/rearrangement of IgG-Fc/sFcγRIIb. The excess entropy change (Table IV), which gives an indication of the number of amino acid residues involved in an induced fit or conformational change

upon binding (41), was estimated from entropy change from the hydrophobic effect (ΔS_{HE}) at T_s subtracted by the entropy contribution due to the loss of rotational and translational degrees of freedom (ΔS_{rt}). The excess entropy for (MN2F)₂ was notably increased, relative to the negligible values for native Fc, (NGA2F)₂, and (M3N2F)₂. This result suggests that folding is coupled to the complex formation of (MN2F)₂ with sFcγRIIb where the estimated value of $\Delta S_{\text{HE}}(T_s)$ is much larger than the magnitude of $\Delta S_{\text{HE}}(T_s)$ for rigid-body associations and ΔS_{rt} . We interpreted this result as an increased C_{H2} hinge proximal disorder for the uncomplexed (MN2F)₂ glycoform: complex formation with sFcγRIIb now requires a conformational change within the C_{H2} domains, leading to a more negative ΔC_p value. The modulation of sFcγRIIb-IgG-Fc interactions by truncation of the oligosaccharides of Fc corresponds to our previous proposal that oligosaccharide-protein interactions of the C_{H2} domains result in a dynamic equilibrium between multiple conformers, certain of which express interaction sites specific to individual ligands (2). It is apparent that truncated oligosaccharides of IgG-Fc retain oligosaccharide/C_{H2} domain interactions and that the resulting glycoforms exhibit a modulated stability and functionality.

Influence of Deglycosylation—Deglycosylation of IgG-Fc abrogated sFcγRIIb binding (Fig. 3e). The dramatic difference in functional activity that results from deglycosylation of IgG has not been paralleled by demonstrations of large-scale structural changes. A minor structural change in conformation of the C_{H2} domains was revealed by an NMR study in which His-268 was used as a reporter residue (42). Here we showed that the deglycosylated C_{H2} domains of IgG1-Fc (Cri) exhibit the lowest T_m value of the series of glycoforms employed, together with a loss of co-operativity (Fig. 5, Table V), which is essentially the same as the result obtained with IgG1-Fc (Wid) although the allotype is different (8). Interestingly, the behavior of deglycosylated C_{H2} domains (Fig. 5A, iv) is totally different from that of the C_{H3} domains with respect to cooperativity as well as T_m value. The amino acid residues of the C_{H2} domains analogous to those in the C_{H3} domains involved in C_{H3}-C_{H3} contact make contacts with the oligosaccharides (6). Therefore, it is likely that deglycosylation results in C_{H2}-C_{H2} contacts through hydrophobic areas that were originally covered with the oligosaccharides. However, deglycosylated C_{H2} domains do not exhibit cooperativity in contrast to the C_{H3} domains even when released from the constraints imposed by the interchain disulfide bridges (Fig. 5B, ii). Radaev and Sun (43) have proposed that deglycosylation of Fc leads to a change in the relative orientation of the two C_{H2} domains such that there is a transition from an open to a closed conformation. The gradual decrease in affinity of the Fc glycoforms for sFcγRIIb on truncation of the oligosaccharides (Fig. 3, Table III) supports this proposal. In addition to such conformational change, we emphasize that impaired FcγR binding by deglycosylation could be due, in part, to destabilization of the C_{H2} domains (Fig. 5, Table V).

Implications of IgG Glycosylation for Therapeutic Applications—The modulated activity of the (MN2F)₂ glycoform of IgG may have applications for activation or deactivation of effector cells via FcγRs. Remodeling of the oligosaccharide profile of IgG constitutes an alternative approach to modulate the biological activity of therapeutic IgGs in addition to protein engineering. Therapeutic antibodies are currently approved or under clinical trial for the treatment of some types of cancer, infectious diseases, and autoimmune disorders (44). The mouse- and hamster-derived cell lines such as NS0 and Chinese hamster ovary cells can produce human IgG antibodies that express "wild-type" biological activities, however, "mutant" Chinese hamster ovary cells can emerge with defined

defects in carbohydrate biosynthesis. The mutant Chinese hamster ovary cells incapable of processing the high-mannose intermediate (Lec 1), adding sialic acid (Lec 2) or galactose (Lec 8), have been reported (45, 46). The resulting IgG bearing distinct oligosaccharides exhibit different functional properties with respect to complement and FcγRI activation and *in vivo* half-life. Chinese hamster ovary cells do not have a capacity for the addition of bisecting GlcNAc; cell engineering with the introduction of β(1-4)-N-acetylglucosaminyltransferase III gene has been shown to yield antibody products that promoted killing target cells at ~10–20-fold lower concentrations than the parent molecules lacking bisecting GlcNAc (47, 48). On the other hand, aglycosylated anti-CD3 monoclonal antibody has been shown to be a promising candidate for immunosuppressive therapy due to its inability to bind to FcγRs or activate complement (49). Fcγ fragments are also proven immunosuppressants for the treatment of immune thrombocytopenic purpura capable of blocking the Fcγ receptors (50) while peptide inhibitors for the IgG-FcγRs interaction have been developed for modulation of immune response (43, 51). Recently we have shown that truncation of the oligosaccharides of humanized anti-major histocompatibility complex class II (L243-IgG1) results in progressive reduction in stimulation of superoxide production through FcγRI in a monocyte-like cell line (8). The thermodynamic data presented here provide a rationale for the modulation of antibody effector functions through manipulation of glycosylation.

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