Stoichiometry and Thermodynamics of the Interaction between the Fc Fragment of Human IgG_1 and Its Low-Affinity Receptor FcγRIII[†]

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ABSTRACT: IgG-Fc receptors, cell surface glycoproteins binding the Fc region of antibodies, play a crucial role in the immune system. To better understand the nature of the recognition process, we have examined the interaction between huIgG$_1$-Fc and a soluble fragment of huFcγRIII (sCD16). Analytical ultracentrifugation experiments clearly demonstrate that IgG$_1$-Fc and sCD16 interact weakly to form a 1:1 complex with an association constant of 1.7×10^5 M^{-1} in PBS at 22.0 °C. The thermodynamic parameters, obtained from the temperature dependence of the equilibrium binding constants, exhibit an enthalpy−entropy compensation with a favorable enthalpy at physiological temperatures. The value of −360 cal mol^{-1} K^{-1} for $\Delta C_p°$ possibly identifies the process as one in which local folding/rearrangement is coupled to complex formation. The 1:1 stoichiometry and thermodynamic parameters provide a basis for understanding the nature of the FcγR−IgG interactions.

The human IgG-Fc[1] receptors, huFcγR, are a group of three cell surface, class I membrane glycoproteins which bind the IgG antibodies through the constant Fc domain (Kinet, 1989; Ravetch & Kinet, 1991; Unkeless et al., 1988). Designated huFcγRI (CD64), huFcγRII (CD32), and huFcγRIII (CD16), these human FcγR contain extracellular immunoglobulin-like domains involved in the recognition of the IgG antibodies (Hogarth et al., 1992; Ravetch & Kinet, 1991; Unkeless et al., 1988), and together with the homologous high-affinity IgE receptor, FcϵRI, they constitute a subfamily of the immunoglobulin gene superfamily (Williams & Barclay, 1988). The huFcγR are found distributed on various hemopoietic cell types (*e.g.,* macrophages, eosinophils, neutrophils, natural killer cells, and lymphocytes), where they play a vital role in antibody effector functions. Binding of antigen constrained IgGs to the Fc receptor brings about cross-linking of the FcγR, which in turn triggers various effector functions, such as phagocytosis, B cell activation and regulation, cellular degranulation, and ADCC, depending on the receptor and cell type activated (Lin et al., 1994; Ravetch & Anderson, 1990; Sandor & Lynch, 1993). Characterization of the IgG−huFcγR interactions may thus lead to a better understanding of the mechanism of IgG−huFcγR recognition and subsequent signal generation and transduction.

The association of the high-affinity huFcγRI with IgG has been characterized, and an association constant K_a of 10^8−10^9 M^{-1} has been determined depending on the IgG isotype (Canfield & Morrison, 1991; Ravetch & Kinet, 1991). Excluding the high-affinity IgE receptor, FcϵRI, it is the only Fc receptor for which a binding affinity to the corresponding antibody has been measured. Unlike huFcγRI, only a maximum value K_a of 10^6 M^{-1} has been determined for the low-affinity receptors huFcγRII and huFcγRIII (Lin et al., 1994), and the physical characterization of the IgG−huFcγRII or −huFcγRIII interactions is apparently complicated by this weak association.

Analytical ultracentrifugation is a technique ideally suited for the study of interacting systems involving soluble proteins whose affinity is in the range of 10^6 M^{-1} (Becerra et al., 1991; Lewis & Youle, 1986). In addition to a direct measure of the association constant *in vitro*, sedimentation equilibrium experiments provide information on the stoichiometry of the complex formed between the associating species. This paper describes an *in vitro* study of the interaction between the Fc portion of human IgG$_1$ with a soluble fragment of huFcγRIII, sCD16, comprising the two extracellular domains of the receptor involved in IgG recognition (Scallon et al., 1989). Experiments were performed with IgG$_1$-Fc, rather than whole IgG$_1$, as the buoyant molecular mass for IgG is much larger

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[1] Abbreviations: IgG, immunoglobulin G; IgE, immunoglobulin E; Fc, constant or effector region of immunoglobulins; Fab, antigen binding region of immunoglobulins; FcγR, IgG-Fc receptors; FcϵRI, high-affinity IgE-Fc receptor; FcRn, neonatal Fc receptor; ADCC, antibody-dependent cellular cytotoxicity; NK, natural killer; hu, human; sCD16, soluble extracellular fragment of human FcγRIII; sFcϵRIα, soluble extracellular fragment of the high-affinity IgE receptor α-chain; Cγ2, immunoglobulin G heavy-chain constant region 2; Cγ3, immunoglobulin G heavy-chain constant region 3; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Gal, galactose; Man, mannose; GlcNac, *N*-acetyl glucosamine; Fuc, fucose.

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than that observed for sCD16; this larger difference in the buoyant molecular masses is a potential source of problems in data analysis by mathematical modeling.

Given that simple symmetry considerations indicate that the Fc portion of IgG has the potential to bind a maximum of two FcγR (Deisenhofer, 1981), the primary goal of the experiments was to determine the stoichiometry of the IgG_1-Fc and sCD16 complex. In a series of sedimentation equilibrium experiments, we found that IgG_1-Fc and sCD16 interact weakly and reversibly *in vitro* to form a complex with a 1:1 stoichiometry. A complete thermodynamic characterization of the association may lead to an increased understanding of the molecular details of the interaction of IgG_1-Fc and sCD16. Therefore, advantage was taken of the relatively weak and reversible association to characterize the thermodynamics of the interaction, through a temperature-dependent measurement of K_a. The data presented in this paper suggest a mechanism by which the specific recognition of the IgG_1-Fc by sCD16 leads to the formation of a 1:1 complex.

We also describe a simple method to determine the carbohydrate content of a glycoprotein by analytical ultracentrifugation.

EXPERIMENTAL PROCEDURES

Preparation of IgG_1-Fc and sCD16. IgG_1-Fc was prepared from human myeloma plasma IgG_1 (Calbiochem) using an ImmunoPure Fab Kit (Pierce). Briefly, the IgG_1 was digested into Fab and Fc fragments using papain, as directed in the kit. Undigested IgG_1 and IgG_1-Fc were separated from the Fab fragments by immobilization on a protein A column and eluted using ImmunoPure elution buffer. IgG_1-Fc was purified from any undigested IgG_1 on a Superdex HR75 10/30 gel filtration column (Pharmacia) using PBS and 0.05% sodium azide as an elution buffer [8.1 mM Na_2HPO_4, 140 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, and 0.7 mM NaN_3 (pH 7.4)]. sCD16 was prepared as described, and the receptor was affinity purified on an IgG 3G8-Sepharose column (Szegedi et al., 1993). The purity and molecular mass of both the IgG_1-Fc and sCD16 preparations were verified by reducing SDS−PAGE and analytical ultracentrifugation. The concentrations of the IgG_1-Fc and sCD16 solutions were determined using calculated extinction coefficients of 69 360 and 46 080 M^{-1} cm^{-1} at 280 nm, respectively, based on the amino acid composition (Wetlaufer, 1962).

Analytical Ultracentrifugation. Analytical ultracentrifugation was performed using a Beckman XL-A analytical ultracentrifuge. Data were acquired as an average of 25 absorbance measurements at a wavelength of 280 nm and a radial spacing of 0.001 cm. All sedimentation equilibrium experiments were run in PBS and 0.05% sodium azide, using double-sector charcoal filled epon centerpieces and column lengths of ~4 mm. The density of the solvent was measured at 20.00 °C (Anton Parr DMA58 density meter) and corrected using standard tables. Sedimentation equilibrium experiments on IgG_1-Fc and sCD16 were conducted at various temperatures, from 4.0 to 28.0 °C, and at 16 000 rpm using loading concentrations corresponding to a measured A_{280} of ~0.5. The time required for the attainment of equilibrium was established by running at a given rotor speed until successive scans, taken 12 h apart, were invariant. Data were then analyzed to obtain the buoyant molecular mass, $M(1 - \bar{v}\varrho)$, using the Optima XL-A data analysis software (Version 2.0, Beckman) running under Microcal Origin 2.8, by fitting data from each scan to

$$A_r = \exp(\ln(A_o) + HM(1 - \bar{v}\varrho)(r^2 - r^2_o)) + E \quad (1)$$

where A_o is the absorbance at a reference point r_o, A_r is the absorbance at a given radial position r, H represents $\omega^2/2RT$, ω the angular speed (in rad s^{-1}), R is the gas constant, T is the absolute temperature, and E is a small baseline correction term. Residuals were calculated by subtracting the best fit of eq 1 from the experimental data. In all cases, a random distribution of the residuals around zero was noted as a function of the radius.

Experiments to measure the equilibrium association constants were carried out at a rotor speed of 16 000 rpm and temperatures in the range of 1.0−25.0 °C. Solutions containing 1:2, 1:1, and 2:1 molar mixtures of IgG_1-Fc−sCD16 with a combined A_{280} of ~0.5 were loaded in each cell and cooled to 1.0 °C. The time required for the attainment of equilibrium at this temperature was 48 h. After data collection at equilibrium, the temperature was increased by 3.0 °C and scans were taken at 12 h intervals. Equilibrium at each temperature was attained within 24 h. Following scanning, the temperature was increased by a further 3.0 °C, as before, and repeated until a temperature of 25.0 °C was attained. Three different loading ratios of IgG_1-Fc and sCD16 were used because it has been demonstrated that the simultaneous fitting of data from such samples represents a stringent criterion for establishing the validity of the assumption that the system is indeed a reversibly associating system (Roark, 1976). Data analyses by mathematical modeling were performed using Sigma Plot 4.16 (Jandel Scientific, San Rafael, CA) operating on a Quadra 700 Macintosh computer. Simultaneous weighted nonlinear least-squares fitting of the data sets at each temperature was performed using different mathematical models of the following form:

$$\begin{aligned} A_r = {} & A_{o,A} \exp(HM_A(r^2 - r^2_o)) + A_{o,B} \times \\ & \exp(HM_B(r^2 - r^2_o)) + A_{o,A} A_{o,B} \times \\ & \exp(\ln k_1 + H(M_A + M_B)(r^2 - r^2_o)) + A_{o,A} (A_{o,B})^2 \times \\ & \exp(\ln k_2 + H(M_A + 2M_B)(r^2 - r^2_o)) + E \end{aligned} \quad (2)$$

where $A_{o,A}$ is the absorbance of the IgG_1-Fc at a reference point r_o, and $A_{o,B}$ is the absorbance of the sCD16 at the reference point r_o. The values of M_A and M_B represent the experimentally determined buoyant molecular masses for the IgG_1-Fc and sCD16, respectively. The equilibrium constants for the 1:1 association, k_1, and the 1:2 association, k_2, of IgG_1-Fc with sCD16 are on an absorbance concentration scale. A_r, H, and E have been previously defined. These models have the equilibrium constant(s) as global fitting parameter(s) and cell reference concentrations and baseline corrections as local fitting parameters.

Sigma Plot employs the Levenberg−Marquardt algorithm (Marquardt, 1963) for the nonlinear, iterative curve fitting, which returns the best-fit parameters and their corresponding asymptotic standard errors. Near convergence, the locally linearized model allows for the use of the Gauss−Newton equations to estimate standard errors of the fitting parameters

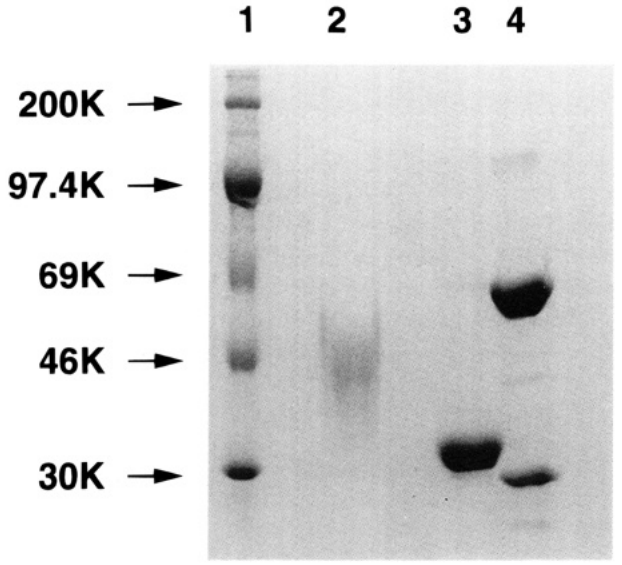

FIGURE 1: Reducing SDS−10% PAGE of human IgG_1, IgG_1-Fc, and sCD16: (lane 1) molecular mass markers with corresponding molecular masses indicated (in kDa); (lane 2) sCD16; (lane 3) $huIgG_1$-Fc; (lane 4) $huIgG_1$ (heavy and light chains).

based on the Jacobian and residual sum of squares (Nash & Walker-Smith, 1987). The equations used by Sigma Plot for the estimation of the parameter's asymptotic standard error (Jennrich, 1977) assume that the model is pseudolinear. Accordingly, the standard errors can only be a true approximation of the best value if the criterion of model pseudolinearity is satisfied.

Thermodynamic Analysis. The values of ln k_1 obtained experimentally were converted to ln K_a values, K_a now being the association constant on a molar scale, using the extinction coefficients calculated for the IgG_1-Fc (ϵ_A) and sCD16 (ϵ_B). Assuming that the extinction coefficient of the complex is the sum of the extinction coefficients of the components, it can be shown that for a given path length l (in cm)

$$\ln K_a = \ln k_1 + \ln(\epsilon_A \epsilon_B l/(\epsilon_A + \epsilon_B)) \qquad (3)$$

The corresponding free energies were then calculated:

$$\Delta G^\circ_T = -RT \ln K_a \qquad (4)$$

Assuming that the change of the heat capacity for the association, ΔC_p° is a temperature-independent variable, it can be shown that (Yoo & Lewis, 1995)

$$\Delta G^\circ_T = \Delta H^\circ_{T_o} - T\Delta S^\circ_{T_o} + (T - T_o)\Delta C_p^\circ - T\Delta C_p^\circ \ln(T/T_o) \qquad (5)$$

Experimental ΔG°_T values were fitted to eq 5 with a reference temperature T_o of 273.15 K to obtain $\Delta H^\circ_{T_o}$, $\Delta S^\circ_{T_o}$, and ΔC_p°.

RESULTS

Characterization of IgG_1-Fc and sCD16. The purity of the preparations of IgG_1-Fc and sCD16 was assessed by SDS−PAGE (Laemmli, 1970) (Figure 1). The IgG_1-Fc migrated as a single band with an apparent molecular mass of 32 000 g mol^{-1}, whereas the preparation of sCD16 migrated as a single broad zone spanning the apparent molecular mass range of 42 000−48 000 g mol^{-1}. The samples were further characterized by sedimentation equilibrium experiments at 22.0 °C which yield the buoyant molecular mass $M(1 - \bar{v}\varrho)$ (Table 1). The protein samples are monodisperse, within the experimental precision of the method, as identical values of $M(1 - \bar{v}\varrho)$ were obtained at different rotor speeds.

In order to obtain the corresponding molecular mass for these glycoproteins, the partial specific volume $\bar{v}$ is required. While insufficient material was available for an experimental determination of $\bar{v}$, both the molecular mass and the unknown carbohydrate content can be readily obtained based on the experimental $M(1 - \bar{v}\varrho)$. Consider a glycoprotein of molecular mass M, with a known amino acid composition corresponding to a protein molecular mass contribution of M_p. The partial specific volume of the protein component, $\bar{v}_p$, is calculated from the amino acid composition (Perkins, 1986), and a calculated or average value for the partial specific volume of the carbohydrate component ($\bar{v}_c$) is assumed. The additivities of the partial specific volumes (Durchschlag, 1986) and molecular mass contributions implies additivity of the buoyant molecular masses:

$$M(1 - \bar{v}\varrho) = M_c(1 - \bar{v}_c\varrho) + M_p(1 - \bar{v}_p\varrho) \qquad (6)$$

Substituting $M - M_p$ for the carbohydrate molecular mass, M_c, one obtains

$$M(1 - \bar{v}\varrho) = M(1 - \bar{v}_c\varrho) + M_p(\bar{v}_c - \bar{v}_p)\varrho \qquad (7)^2$$

Thus, the experimentally determined value of $M(1 - \bar{v}\varrho)$ leads to a unique solution for M, from which one obtains M_c as the difference between M and M_p.

The IgG_1-Fc possesses two glycosylation sites known to be occupied (Deisenhofer, 1981; Sutton & Phillips, 1983). Assuming a consensus complex biantennary sequence of $Gal_2Man_3GlcNAc_4Fuc_1$ for each site, a value of $\bar{v}_c$ of 0.650 cm^3 g^{-1} is calculated (Durchschlag, 1986). The experimentally determined value of $M(1 - \bar{v}\varrho)$ for IgG_1-Fc, the calculated value of $\bar{v}_c$, and the known amino acid composition for this constant domain (Cunningham et al., 1970; Ellison et al., 1982; Gall & Edelman, 1970; Ponstingl & Hilschmann, 1976; Rutishauser et al., 1970; Schmidt et al., 1983) yield a molecular mass of 52 300 ± 500 g mol^{-1}, in reasonable agreement with the expected molecular mass of 53 226 g mol^{-1} (Table 1).

The amino acid sequence of sCD16 (Scallon et al., 1989) reveals five putative N-glycosylation sites (Asn-X-Thr/Ser). Assuming that the carbohydrate chains at each site are of the complex biantennary type, with a core composition of $Gal_2Man_3GlcNac_4$ (Kornfeld & Kornfeld, 1985), then a $\bar{v}_c$ of 0.649 cm^3 g^{-1} is calculated. Thus, the experimental $M(1 - \bar{v}\varrho)$ leads to a molecular mass of 31 000 ± 400 g mol^{-1}, corresponding to 24% (w/w) carbohydrate (Table 1). This is slightly less than the calculated molecular mass of 31 565 g mol^{-1} and corresponds to a 93% occupancy of the potential N-glycosylation sites.

To further characterize the IgG_1-Fc and sCD16, the buoyant molecular masses were determined as a function of the temperature and reduced to the corresponding partial specific volumes using the experimentally calculated values

[2] The methodology described above was developed independently by M. S. Lewis and R. P. Junghans (submitted for publication) and by H. J. Gould and co-workers (Keown et al., 1995).

Table 1: Characterization of IgG_1-Fc and sCD16 by Sedimentation Equilibrium

sample	$M(1 - \bar{v}\varrho)^a$/g mol^{-1}	$M_p{}^b$/gmol^{-1}	$\bar{v}_p{}^b$/cm^3 g^{-1}	$M_r{}^c$/g mol^{-1}	M^d/g mol^{-1}
IgG1-Fc	13 900 ± 200	49 690	0.736	52 300 ± 500	53 226
sCD16	8 700 ± 150	23 455	0.737	31 000 ± 400	31 565

[a] Experimentally determined buoyant molecular weight at 22.0 °C. The error is the standard deviation of the mean, based on four measurements at three rotor speeds, ranging from 12 000 to 16 000 rpm. [b] Molecular weight of the protein component and the corresponding partial specific volume (Perkins, 1986). [c] Experimentally calculated molecular weight based on eq 7, as described in the text. [d] Calculated molecular weight based on the known amino acid composition and a complete carbohydrate complement.

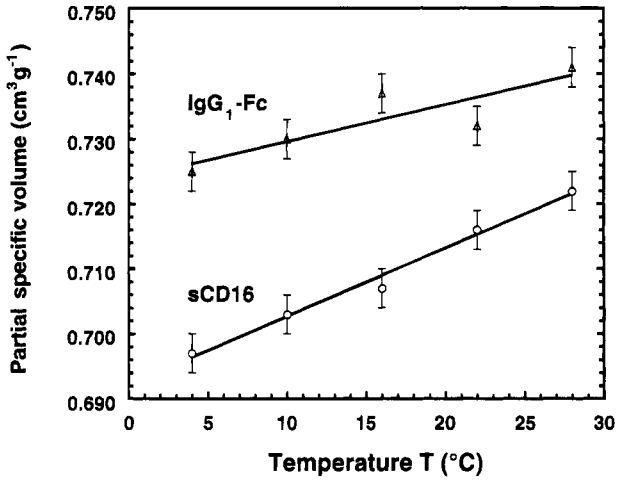

FIGURE 2: Experimentally determined values of $\bar{v}$ for IgG_1-Fc and sCD16 in PBS and 0.05% NaN_3, as a function of the temperature, calculated from the concentration distribution at equilibrium at a rotor speed of 16 000 rpm. The loading concentrations of each sample was $0.5A_{280}$. The data at 4.0 and 22.0 °C are an average of four measurements at three different rotor speeds ranging from 12 000 to 16 000 rpm, for both the IgG_1-Fc and the sCD16. In all cases, the error is set to a nominal value of ± 0.003 cm^3 g^{-1}, based on the standard error of the buoyant molecular mass measured at 22.0 °C (Table 1). The best-fit straight lines to the data are as follows: $\bar{v} = 0.724 + (5.67 \times 10^{-4})T$ (°C) ($R^2 = 0.87$) for IgG_1-Fc, and $\bar{v} = 0.692 + (10.5 \times 10^{-4})T$ (°C) ($R^2 = 0.99$) for sCD16.

of the molecular masses (Table 1). In the temperature range of 4.0−28.0 °C, $\bar{v}$ appears to vary linearly with temperature for both samples (Figure 2). A linear dependence of $\bar{v}$ with temperature has been observed for a number of proteins [reviewed by Durchschlag (1986)] with an average $d\bar{v}/dT$ of 4.25×10^{-4} cm^3 g^{-1} K^{-1}. In the case of the IgG_1-Fc and sCD16, the corresponding $d\bar{v}/dT$ values obtained experimentally are $(5.7 \pm 0.2) \times 10^{-4}$ and $(10.5 \pm 0.7) \times 10^{-4}$ cm^3 g^{-1} K^{-1}, respectively. These are larger than typical $d\bar{v}/dT$ values, yet within the range of values reported for various proteins (Durchschlag, 1986).

IgG_1-Fc and sCD16 Interaction To Form a 1:1 Complex. In order to establish the stoichiometry of the IgG_1-Fc and sCD16 complex, a series of sedimentation equilibrium experiments were carried out on mixtures of the two components. Three different loading ratios of IgG_1-Fc−sCD16, corresponding to 1:2, 1:1, and 2:1 were studied simultaneously at various temperatures.

The sedimentation equilibrium data from the three different loading ratios were fitted simultaneously to various models, based on the formation of 1:1 or 1:2 IgG_1-Fc−sCD16 complexes. The data were initially fitted to a model in which the IgG_1-Fc and sCD16 interact to form a 1:1 complex, corresponding to eq 2 having the last term containing ln k_2 removed. This model yields an excellent fit to the data at all temperatures, returning a ln K_a values of 12.7 ± 0.04 and 12.0 ± 0.02 at 4.0 and 22.0 °C, respectively. Figure 3A shows the best fit to the data using this model at 22.0 °C; the corresponding residuals (Figure 3B) are typical of those obtained at various temperatures and illustrate the validity of this model. The data were also fitted using a

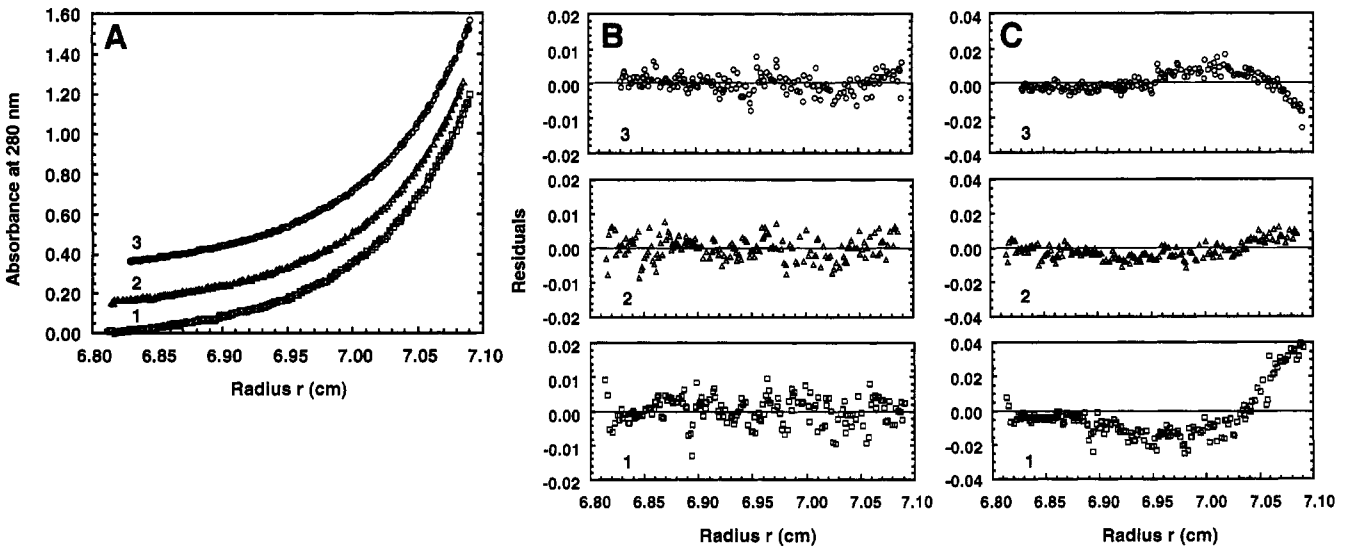

FIGURE 3: Sedimentation equilibrium profiles at 280 nm, and the corresponding residuals, for mixtures of IgG_1-Fc and sCD16 at 16 000 rpm and 22.0 °C. The symbols correspond to the following ratios of IgG_1-Fc to sCD16: 1, (squares) 1:2; 2, (triangles) 1:1; 3, (circles) 2:1. The initial concentrations corresponded to a combined A_{280} of 0.5. (A) Sedimentation equilibrium profiles at 280 nm. The data for 1 have been shifted by $-0.1A_{280}$, and that for 3 by $+0.2A_{280}$ for clarity. The lines through the data are the best fit for a reversible 1:1 association. (B) Distribution of the residuals for the 1:1 interaction between IgG_1-Fc and sCD16. (C) Distribution of the residuals for the 1:2 interaction between IgG_1-Fc and sCD16.

model in which the IgG_1-Fc and sCD16 associate to form a single complex having a 1:2 stoichiometry, with both of the potential sCD16 binding sites occupied. This corresponds to eq 2 with removal of the third term containing ln k_1. At 22.0 °C, the best fit returns residuals that are clearly not randomly distributed (Figure 3C) and a corresponding minimum sum of squares that is much higher than that obtained for the 1:1 association. Similar patterns for the residual distributions and minimum sum of squares were obtained at the various temperatures studied, indicating that the 1:1 association is a correct model.

The general case in which both 1:1 and 1:2 IgG_1-Fc–sCD16 complexes are formed was also considered (eq 2). In the range of 1.0–13.0 °C, the fit returns ln k_1 values identical to those obtained for the 1:1 association and ln k_2 values less than −40, demonstrating that the IgG_1-Fc and sCD16 interact to form only a 1:1 complex when mixed at concentrations under consideration (~5 μM). However, the data in the range of 16.0–25.0 °C are consistent with the formation of both 1:1 and 1:2 complexes. The corresponding K_a values for the 1:1 association are ~10% smaller than the values obtained in the model where only the 1:1 complex is formed; the K_a value for the 1:2 association is of the order of 1.0×10^9 M^{-2} in all these cases. The distributions of the residuals are random, and the minimum sum of squares returned is only less than a 5% improvement over the 1:1 model. Indeed, the fact that one can model the higher temperature data as a combination of the formation of both 1:1 and 1:2 complexes is probably a consequence of the weak binding interaction observed. Since an increased number of fitting parameters invariably yields a better fit, the improvement in the minimum sum of squares is negligible, and the fact that at lower temperatures the data are consistent with the formation of only a 1:1 complex, we propose that these data support the formation of only a 1:1 complex even at the higher temperature range considered.

If a mathematical model is a linear function of the fitting parameters, and if the data have normally distributed residuals, then a plot of the sum of squares as a function of the parameter of interest will be a parabola symmetric about the parameter best-fit value (Lewis et al., 1994). In the case of the 1:1 association of IgG_1-Fc and sCD16, we have found that the curve describing the sum of squares returned as a function of the value of ln k is symmetrically parabolic to within ±3 standard errors returned from the fit, thus exceeding the 95% confidence limit of ±2 standard errors. Furthermore, the residuals of the best fit (Figure 3B) were found to be normally distributed (data not shown). Therefore, the 1:1 association model and experimental data constitute a pseudolinear system, justifying the use of the standard error returned as the best estimate thereof (Lewis et al., 1994). Note that the standard error in lnK_a does not account for uncertainties in the extinction coefficients of the IgG_1-Fc and sCD16 or the error in the determination of their respective buoyant molecular weights.

Thermodynamics of the Association. Having established the fact that the IgG_1-Fc and sCD16 associate to form a 1:1 complex, all of the data were analyzed in terms of this model. Using the experimentally observed linear dependence of $\bar{v}$ for both the IgG_1-Fc and sCD16 (Figure 2) and the experimentally determined molecular masses (Table 1), the corresponding values of $M(1 - \bar{v}\rho)$ for both components at each temperature were computed. The corresponding values

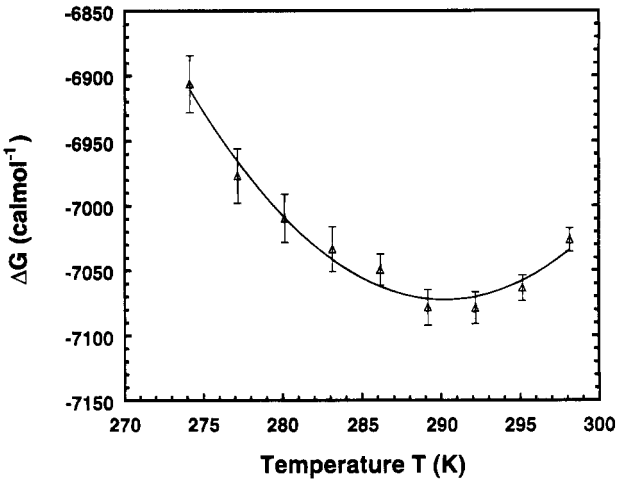

FIGURE 4: Temperature dependence of the free energy $\Delta G°_T$ for the 1:1 association of IgG_1-Fc and sCD16. The error bars represent the estimated standard error returned from fitting the experimental data. The $\Delta G°_T$ data were fit to eq 5 with a reference temperature T_o of 273.15 K to yield the parameters in Table 2 and the best-fit line shown.

Table 2: Thermodynamic Parameters for the 1:1 Association of IgG_1-Fc and sCD16

parameter	$T = 273.15$ K[a]
$\Delta H°$/kcal mol^{-1}	-1.0 ± 0.5
$\Delta S°$/cal mol^{-1} K^{-1}	22 ± 2
$\Delta C_p°$/cal mol^{-1} K^{-1}	-360 ± 40
$\Delta G°$/kcal mol^{-1}	-7.0 ± 1.0

[a] The values of $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ are obtained from eq 5 with a T_o of 273.15 K. The errors represent the estimated standard error obtained from the data fitting.

of the association constant, K_a, and the free energy, $\Delta G°_T$ were then obtained. K_a was found to decrease with temperature from a nominal value of 3.2×10^5 M^{-1} at 1.0 °C to 1.4×10^5 M^{-1} at 25.0 °C, as evidenced by the corresponding values of $\Delta G°_T$ (Figure 4).

These data were fitted to eq 5 to yield the thermodynamic parameters characterizing the 1:1 association (Table 2); the corresponding data fit is shown in Figure 4. These parameters are derived on the assumption that the change of the heat capacity for the association, $\Delta C_p°$, is temperature independent, an assumption tested by fitting the data to an expanded form of eq 5 based on a linear temperature dependence of $\Delta C_p°$ (Yoo & Lewis, 1995). The parameters returned are identical to those in Table 2 and the value of the additional $d\Delta C_p°/dT$ term is negligible, justifying the use of eq 5. Interestingly, the thermodynamics are typical of an entropy–enthalpy compensation process with $|\Delta C_p°| \gg |\Delta S°|$, and the association is characterized by a favorable enthalpic contribution over the temperature range studied. The significant negative $\Delta C_p°$ possibly identifies the association process as one in which local folding/rearrangement is coupled to complex formation (Spolar & Record, 1994). The entropic contribution is favorable at low temperatures and becomes unfavorable above a T_S of 290 K, at which the entropic contribution is essentially zero. The corresponding temperature at which the enthalpic contribution is zero, T_H, is 270 K.

DISCUSSION

Determination of Carbohydrate Content. The measured value of the molecular mass of the IgG_1-Fc is 1000 g mol^{-1}

smaller than the expected molecular mass, assuming that both the Asn 297 residues contain the full consensus carbohydrate sequence of composition $Gal_4Man_6GlcNAc_8Fuc_2$. It has been shown (Jefferis, 1993; Lund et al., 1993), however, that there is a profile of related oligosaccharide structures found on the Fc portion of human IgG_1 and other human IgGs, rather than a single consensus composition, and the majority (>80%) of the oligosaccharide structures found on the IgG_1 class contain a complex biantennary structure having a minimal composition of $Man_6GlcNAc_8Fuc_2$ with either zero, one, or two capping galactose moieties. In the case of a paucidisperse system, such as the IgG_1-Fc under consideration, the measured molecular mass represents a weight average molecular mass (Tanford, 1961); based solely on the data presented by Lund and co-workers (1993), a weight average molecular mass of 52 900 g mol^{-1} is calculated, which is within the error of that calculated experimentally. Note that since the value of $\bar{v}_c$ is insensitive to small changes in the carbohydrate composition, a direct comparison of the calculated weight average molecular weight and the experimentally determined values can be made.

Assuming a $\bar{v}_c$ of 0.649 cm^3 g^{-1}, based on the expected core composition of $Gal_2Man_3GlcNac_4$ (Kornfeld & Kornfeld, 1985) for N-glycosylation sites, the experimentally determined buoyant molecular mass for sCD16 returns a molecular mass of 31 000 ± 400 g mol^{-1}. This value is slightly smaller than that calculated for sCD16 containing a full complement of carbohydrate on each of the five potential glycosylation sites and corresponds to a 93% occupancy. Thus, for both the IgG_1-Fc and sCD16, the experimentally calculated molecular masses are identical (within experimental error) to those expected (Table 1), demonstrating the validity of the method (eq 7) for the determination of the molecular mass, as well as the homogeneity of the samples. Note that this method for the determination of the molecular mass and carbohydrate content makes no assumptions about the potential number of glycosylation sites; only a reasonable value for $\bar{v}_c$ need be assumed, making this technique useful as a general method for the analysis of glycoproteins and the determination of carbohydrate content.

The experimentally determined partial specific volumes for both the IgG_1-Fc and sCD16 vary linearly with temperature in the range of 4.0−28.0 °C (Figure 2), yielding slopes ($d\bar{v}/dT$), which are larger than the average $d\bar{v}/dT$ observed for nonglycosylated proteins (Durchschlag, 1986). This increased value of $d\bar{v}/dT$ is presumed to result from the carbohydrate portion of the glycoproteins, especially since increasing glycosylation appears to result in larger $d\bar{v}/dT$ values (*cf.* IgG_1-Fc *versus* sCD16). Assuming that $d\bar{v}/dT$ is only a reflection of the change in the partial specific volume and that the protein and carbohydrate portion expand independently of each other with increasing temperature, it is possible to estimate a value for $d\bar{v}_c/dT$, the change in the carbohydrate partial specific volume as a function of temperature. Additivity of the partial specific volumes (Durchschalg, 1986) combined with the above assumptions, yields

$$M(d\bar{v}/dT) = M_c(d\bar{v}_c/dT) + M_p(d\bar{v}_p/dT) \qquad (8)$$

Using an average value for $d\bar{v}_p/dT$ at 4.25 × 10^{-4} cm^3 g^{-1} K^{-1} (Durchschlag, 1986), combined with the experimentally determined values of $d\bar{v}/dT$ (Figure 2), M, and M_c (Table 1), both the IgG_1-Fc and sCD16 data yield identical values of $d\bar{v}_c/dT$ (within experimental error) averaged at (3.2 ± 0.3) × 10^{-3} cm^3 g^{-1} K^{-1}. On the basis of these simple considerations, it is thus expected that $d\bar{v}_c/dT$ is generally 1 order of magnitude larger than the average $d\bar{v}_p/dT$ value. Simply, the coefficient of thermal expansion for the carbohydrate chain of the glycoprotein is much larger than that for the protein.

Stoichiometry of the Antibody−Receptor Complex. Sedimentation equilibrium data for mixtures containing 2:1, 1:1, and 1:2 IgG_1-Fc−sCD16, in the concentration range of 4.5−3.2 μM IgG_1-Fc and 2.2−6.4 μM sCD16, clearly demonstrate that these species interact weakly to form a 1:1 complex. This result is valid in the temperature range of 1.0−25.0 °C, and independent of the exact loading ratios of IgG_1-Fc and sCD16. Furthermore, since the value of the buoyant molecular mass was determined for both species under essentially identical experimental conditions, or interpolated at certain temperatures based on the experimental data in Figure 2, no assumptions about the molecular mass, partial specific volumes, and solvent densities were made in reaching this stoichiometry.

The 1:1 stoichiometry is identical to that observed between IgE-Fc (Keown et al., 1995) and IgE (Robertson, 1993), with the corresponding high-affinity receptor, FcεRI, yet different from the 2:1 stoichiometry reported for complexes formed between protein A (Deisenhofer, 1981) and FcRn (Burmeister et al., 1994; Huber et al., 1993) with IgG-Fc. On the basis of the crystal structure of the complexes, both protein A (Deisenhofer, 1981) and FcRn (Burmeister et al., 1994) bind to the IgG-Fc at the interface between the Cγ2 and Cγ3 domains in a manner that the occupancy of one site on the IgG-Fc does not occlude the symmetrically related second site. Unlike protein A and FcRn, there is evidence suggesting that the various FcγR interact with the IgG antibodies in the hinge region (Canfield & Morrison, 1991; Duncan et al., 1988; Jefferis et al., 1990; Lund et al., 1990, 1991; Sármay et al., 1985). This is a region where the symmetrically related Cγ2 domains of IgG come into close spatial proximity, and a possible explanation for the 1:1 stoichiometry of the IgG-Fc and receptor complex is steric, whereby the binding of one receptor molecule hinders the binding of a second.

Direct evidence demonstrating that the lower hinge region on the Cγ2 domain of IgG-Fc is involved in the interaction with the various FcγR comes from site-directed mutagenesis studies, where it has been shown that the residues in the lower hinge region comprising Leu 234−Gly 237, and other residues on the proximal bend of IgG_1, are critical for recognition of the high-affinity receptor huFcγRI (Canfield & Morrison, 1991; Duncan et al., 1988; Lund et al., 1991). Additional evidence comes from studies with aglycosylated and intact IgG_3, which fails to yield ADCC (Lund et al., 1990), a cellular response directly linked to activation of huFcγRIII in NK cells. It has been proposed that the absence of carbohydrate at Asn 297 leads to a small and localized protein structural change in the vicinity of the lower hinge region (Lund et al., 1990; Matsuda et al., 1990), identifying this as a potential binding site for huFcγRIII. Indeed, the area near the lower hinge region on the Cγ2 domain has been directly implicated in the recognition of FcγRIII as it has been reported that the FcγR involved in ADCC on human NK cells interacts directly with a stretch of residues from Lys 274 to Glu 294 on the Cγ2 domain of IgG-Fc (Sármay

et al., 1985). Consequently, it has been proposed that this region, wherein the symmetrically related Cγ2 domains come into close proximity, is important in the recognition of all of FcγRI, FcγRII, and FcγRIII (Jefferis et al., 1990), supporting a steric mechanism for the 1:1 stoichiometry observed for the IgG_1-Fc and sCD16 complex.

Steric interference could account for the inability of the IgG to bind two molecules of receptor but is not necessarily the only possible explanation. An alternative mechanism whereby the binding of the first receptor molecule leads to a conformational change and/or structural ordering on the IgG that renders the second binding site nonfunctional may be possible. In this context, it is interesting to note that the lower hinge region of the IgG-Fc fragment, implicated in sCD16 recognition, is found to be disordered in the crystal structure. No interpretable electron density was found for residues Thr 222−Gly 237 (Deisenhofer, 1981), a region encompassing Leu 234−Gly 237 necessary for FcγR recognition (Canfield & Morrison, 1991; Duncan et al., 1988; Lund et al., 1991). The binding of a sCD16 molecule may therefore induce a conformational change or ordering of the lower hinge region structure, resulting in the loss of the second receptor binding site. Indeed, the segmental flexibility of IgG_1, arising from restricted rotational motion of the Fab arms about the hinge, (Dangl et al., 1988; Reidler et al., 1982) is apparently constrained upon binding to huFcγRI (Zheng et al., 1992). Thus, based on the sequence similarities between the IgG-Fc binding immunoglobulin domains of the different huFcγR (Lin et al., 1994; Ravetch & Kinet, 1991; Sandor & Lynch, 1993), it is possible that huFcγR binding to the Cγ2 domain induces a loss of hinge flexibility. This provides evidence supporting a conformational mechanism for the observed stoichiometry. Overall, the 1:1 stoichiometry of the IgG_1-Fc and sCD16 complex can be rationalized in terms of a steric hindrance to a second receptor molecule, a conformational change on the IgG_1-Fc induced by receptor binding, or a combination of both mechanisms. The thermodynamic data presented below support the conformational change mechanism.

Induced-Fit Interaction between Antibody and Receptor. The association constant characterizing the 1:1 association of IgG_1-Fc and sCD16 has experimentally determined nominal values of 3.2×10^5 M^{-1} at 1.0 °C and 1.7×10^5 M^{-1} at 22.0 °C. These data are consistent with the low affinity of sCD16 for IgG (Lin et al., 1994) and compare reasonably well with the apparent association constant of $(2.9 \pm 0.4) \times 10^5$ M^{-1} measured for a mouse low-affinity FcγR, with polyclonal IgG (Poglitsch et al., 1991). In the case of a monoclonal mouse IgG_1, a K_a of $(9.3 \pm 0.2) \times 10^5$ M^{-1} was measured at 25 °C; no information on the stoichiometry of the complex is available (Poglitsch et al., 1991). The interaction of FcRn and human IgG_1 has also been studied by isothermal titration calorimetry leading to an apparent K_a of 2×10^6 M^{-1} at 25.5 °C for this 1:2 IgG_1−FcRn association (Huber et al., 1993).

The temperature dependence of the association of $huIgG_1$-Fc and sCD16 yields thermodynamic parameters characterizing this association. Interestingly, this association is characterized by a negative $\Delta C_p°$, with the enthalpic and entropic contributions to $\Delta G°$ varying with temperature in a nearly parallel fashion. Consequently, the overall value of $\Delta G°$ is nearly constant with temperature; the temperatures at which the enthalpic and entropic contributions to $\Delta G°$ are zero, correspond to T_H = 270 K and T_S = 290 K, respectively. The change in the heat capacity on association, $\Delta C_p°$ can be interpreted in terms of burial of both the polar and nonpolar surface residues upon association. The negative $\Delta C_p°$ indicates that complex formation is accompanied by a larger contribution from the burial of nonpolar residues. However, both the magnitude of $\Delta C_p°$ and the relatively low value of T_S cannot be interpreted solely in terms of changes in polar and nonpolar surface; the association is not necessarily a rigid body association (Spolar & Record, 1994). At T_S, the overall entropic contribution to the association process is zero, and the favorable entropic contribution arising from the hydrophobic effect, approximated by 140 cal mol^{-1} K^{-1} [*i.e.*, $1.35\Delta C_p° \ln(T_S/386)$; see Spolar and Record (1994)], is not wholly compensated for by the unfavorable contribution arising from the loss of rotational and translational degrees of freedom (~50 cal mol^{-1} K^{-1}), necessitating a contribution to ΔS from an induced conformational change or structural ordering. Therefore, the combined negative $\Delta C_p°$ and T_S are consistent with the suggestion that the interaction of sCD16 with IgG_1-Fc induces a local ordering or conformational change, presumably of the apparently disordered lower hinge region of the Cγ2 domain (Deisenhofer, 1981) implicated in FcγR recognition (Canfield & Morrison, 1991; Duncan et al., 1988; Lund et al., 1991). The decreased hinge flexibility upon FcγR binding (Zheng et al., 1992), combined with the observed 1:1 stoichiometry and thermodynamic data, suggests that the proposed structural ordering or conformational change may be a general mechanism for the association of the various human IgG subclasses with the FcγR.

The experimentally determined K_a have corresponding dissociation constants of the same order of magnitude as the IgG_1 concentration *in vivo*, suggesting that some IgG_1 may be bound monomerically to the cell surface huFcγRIII. The monomerically bound IgG, however, is not sufficient to activate the huFcγRIII response; multiple IgG binding and subsequent receptor cross-linking are required to trigger the appropriate immune response. Indeed, it has been shown that both the low-affinity huFcγRII and huFcγRIII receptors avidly bind IgG complexes (Hogarth et al., 1992, Ravetch & Kinet, 1991, Unkeless et al., 1988).

Comparison to IgE-Fc. The affinity of the IgE−FcεRI interaction is exceptionally high, with a K_a of ~10^{10} M^{-1} (Beavil et al., 1993), a value higher than any known IgG-Fc receptor interaction. Unlike IgG_1, IgE has an extra heavy-chain domain pair (Cε2) and a much reduced segmental flexibility of the Fab arms (Slattery et al., 1985). Nonetheless, the amino acid sequence homologue to the lower hinge region in IgG_1 (Padlan & Davies, 1986), *viz.* the N-terminal sequence in Cε3, has been implicated in the recognition of FcεRI (Beavil et al., 1993), although other regions have also been implicated (Presta et al., 1994). The stoichiometry of the IgE−sFcεRIα (Keown et al., 1995) and IgE−sFcεRIα (Robertson et al., 1993) complexes are both 1:1, like that of the IgG_1−FcγRIII measured in the present work, and may signify a similar underlying recognition mechanism involving a receptor-induced conformational change on the IgE antibodies.

Conclusions. This work describes a physical study of the interaction between an antibody and an Fc receptor by analytical ultracentrifugation, a technique ideally suited for the study of weak binding interactions not accessible using

traditional immunological techniques. As demonstrated by this study, analytical centrifugation allows for a determination of both the association constant and the stoichiometry of the complex. The 1:1 stoichiometry and thermodynamic signature characterizing the IgG_1−Fc and sCD16 interaction provide a basis for understanding the nature of the FcγR−IgG interactions.

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