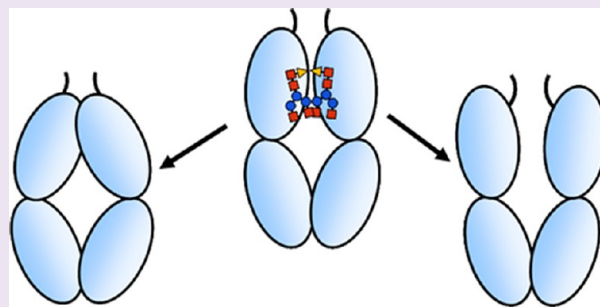


## Revisiting the Role of Glycosylation in the Structure of Human IgG Fc

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**ABSTRACT:** Binding of the Fc domain of Immunoglobulin G (IgG) to Fc $\gamma$  receptors on leukocytes can initiate a series of signaling events resulting in antibody-dependent cell-mediated cytotoxicity (ADCC) and other important immune responses. Fc domains lacking glycosylation at N297 have greatly diminished Fc $\gamma$  receptor binding and lack the ability to initiate a robust ADCC response. Earlier structural studies of Fc domains with either full length or truncated N297 glycans led to the proposal that these glycans can stabilize an “open” Fc conformation recognized by Fc $\gamma$  receptors. We determined the structure of an *E. coli* expressed, aglycosylated human Fc domain at 3.1 Å resolution and observed significant disorder in the C'E loop, a region critical for Fc $\gamma$  receptor binding, as well as a decrease in distance between the C<sub>H</sub>2 domains relative to glycosylated Fc structures. However, comparison of the aglycosylated human Fc structure with enzymatically deglycosylated Fc structures revealed large differences in the relative orientations and distances between C<sub>H</sub>2 domains. To provide a better appreciation of the physiologically relevant conformation of the Fc domain in solution, we determined Radii of Gyration ( $R_g$ ) by small-angle X-ray scattering (SAXS) and found that the aglycosylated Fc displays a larger  $R_g$  than glycosylated Fc, suggesting a more open C<sub>H</sub>2 orientation under these conditions. Moreover, the  $R_g$  of aglycosylated Fc was reduced by mutations at the C<sub>H</sub>2–C<sub>H</sub>3 interface (E382V/M428I), which confer highly selective binding to Fc $\gamma$ RI and novel biological activities.



The Fragment crystallizable (Fc) portion of antibodies provides a link between both the innate and adaptive immune systems as well as between humoral and cellular immunity. Antibodies, whether generated by our own immune systems or administered therapeutically, interact with Fc gamma receptors (Fc $\gamma$ Rs) and complement components (such as C1q) to clear pathogens or to destroy aberrant cells such as tumors.<sup>1,2</sup> Antibodies of the immunoglobulin G (IgG) subclass are composed of two identical Fragment antigen binding (Fab) domains containing variable regions that bind to antigen and a single Fc domain that mediates effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP) via Fc $\gamma$ Rs and complement dependent cytotoxicity (CDC) via C1q.<sup>3</sup>

The horseshoe-shaped, homodimeric Fc domain consists of two C<sub>H</sub>2 domains at the N-terminus and two interacting C<sub>H</sub>3 domains at its C-terminus. Although the two C<sub>H</sub>2 domains do not directly interact, the N-linked glycosylation at N297 bridges the gap between the two domains while shielding the hydrophobic inner surface of C<sub>H</sub>2 from solvent. The N297 glycan consists of a core heptasaccharide (GlcNAc4Man3) chain that can be appended by the addition of fucose at the initial GlcNAc residue or galactose residues at terminal GlcNAc.<sup>4</sup> Additionally, sialic acid residues can also be added to terminal galactose residues. The complete removal of the glycan at N297 greatly reduces binding to effector Fc $\gamma$ Rs expressed by B cells, natural killer (NK) cells, granulocytes, and

monocytes as well as to the complement protein C1q, thus significantly diminishing ADCC and CDC.<sup>5–7</sup> While extensive glycan truncations have been shown to be deleterious to the ability of the Fc domain to interact with Fc $\gamma$ Rs, certain changes in the glycan composition can actually promote favorable effector interactions. Indeed, efforts have focused on the engineering of monoclonal antibodies with glycans optimized for various effector functions.<sup>8,9</sup> For example, defucosylation of the N297 glycan has been reported to enhance binding of Fc $\gamma$ RIIIa and increase NK mediated ADCC by >50-fold.<sup>10–13</sup>

The X-ray structures of four different Fc domains with distinct glycan truncations suggested that shortening of the N297 glycan results in progressively more “closed” conformations, bringing the C<sub>H</sub>2 domains in closer proximity compared to fully glycosylated, “open” Fc conformer.<sup>14</sup> These observations led to the prevailing model that the “open” conformation of glycosylated Fc is critical for physiologically relevant binding to the effector Fc $\gamma$ Rs. Additionally, the crystal structure of glycan truncation variants revealed minor conformational changes within the C'E loops of the Fc domain.<sup>14</sup> As no aglycosylated human Fc structure has yet been described, the exact structural reasons responsible for loss of effector binding affinity in aglycosylated antibodies are unknown. However, a recent structure of an enzymatically deglycosylated murine Fc

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exhibited a “super-closed” state with the C<sub>H</sub>2 domains collapsing toward one another.<sup>15</sup> The observed Fc conformation was consistent with the model that disruption of the open conformation is responsible for the abrogation of FcγR binding and lack of ADCC displayed by aglycosylated Fc domains. It should be noted that unlike the large movements in C<sub>H</sub>2 observed when the glycans are truncated or removed, the C<sub>H</sub>3 domain shows essentially no structural perturbation.

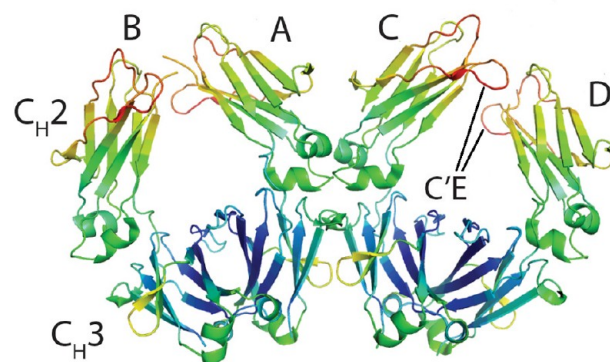
Technical advances in recent years led to the high yield preparative expression of aglycosylated antibodies in bacteria and methods for the isolation of full length IgGs from combinatorial libraries expressed in *E. coli*.<sup>16–18</sup> Several antibodies lacking glycosylation are now under advanced clinical evaluation.<sup>19</sup> For some therapeutic applications the lack of ADCC/CDC exhibited by aglycosylated antibodies is a preferred property, however, in many cases (such as with some antitumor antibodies) ADCC or CDC is desired. Aglycosylated antibodies with restored or tailored ADCC properties are currently under investigation. Recently, the screening of large combinatorial libraries has led to the isolation of aglycosylated Fc variants that bind selectively only to FcγRI<sup>20</sup> or alternatively to the both the activating FcγRIIa and the inhibitory FcγRIIb receptors<sup>21</sup> with high affinity. In particular, the FcγRI selective aglycosylated Fc5 variant, containing two mutations E382V/M428I, was shown to uniquely induce cytotoxicity of target cells with monocyte-derived dendritic cells as effectors, a phenotype not conferred by glycosylated IgG.<sup>20</sup>

The mutations in Fc5 are near the C<sub>H</sub>2–C<sub>H</sub>3 interface and distal to the FcγRI binding epitope.<sup>22,23</sup> This finding raises several questions specifically about the nature of the Fc5:FcγRI interaction and more generally about the importance of the “open” conformation of Fc domains. To address these issues we first solved the structure of a human aglycosylated Fc. Analysis of this and other crystallographic information suggests that the proposed “closed” conformations of aglycosylated Fcs observed in crystals may not be the physiologically relevant form. SAXS analysis further revealed that aglycosylated Fc actually has a larger Radius of gyration ( $R_g$ ) than glycosylated Fc in solution. The aglycosylated Fc5 displays an intermediate  $R_g$  in solution indicating that the degree of “openness” of the aglycosylated Fc can be altered by mutations selected to restore FcγRI binding.

## RESULTS AND DISCUSSION

**Structure of Aglycosylated Human IgG1 Fc.** The structure of human IgG1 Fc purified from *E. coli* and therefore lacking glycosylation was solved at 3.1 Å resolution. The crystal was in the P1 space group with two Fc dimers (composed of polypeptides A,B and C,D, respectively) in the asymmetric unit (Figure 1). The two Fc dimers also interface at the C<sub>H</sub>2–C<sub>H</sub>3 elbow of chains A and C. Phasing by molecular replacement and refinement led to a final model with an *R*-factor of 26.2% ( $R_{\text{free}} = 32.3\%$ ) (Table 1). To reduce structural bias the C<sub>H</sub>2 and C<sub>H</sub>3 domains were treated as separate entities during molecular replacement and refinement, with residues bridging the C<sub>H</sub>2–C<sub>H</sub>3 domains removed from noncrystallographic symmetry restraints.

The four polypeptides (A–D) are composed of residues G236–L443, G236–S442, G237–S444, and P238–S444, respectively, with electron density for amino acids 221–235 (composing the IgG hinge) and 445–447 missing in all chains. Hinge residues in all Fc structures to date have been unobservable in electron density maps presumably due to their high flexibility.<sup>14</sup> Electron density maps were generally



**Figure 1.** Overall structure of aglycosylated IgG Fc domain solved at 3.1 Å resolution. The range of *B*-factors of all *C*as are represented by rainbow-colors ranging from blue (low) to red (high). Two homodimers A,B and C,D are present in the asymmetric unit. The location of the C'E loop is noted in the C,D dimer.

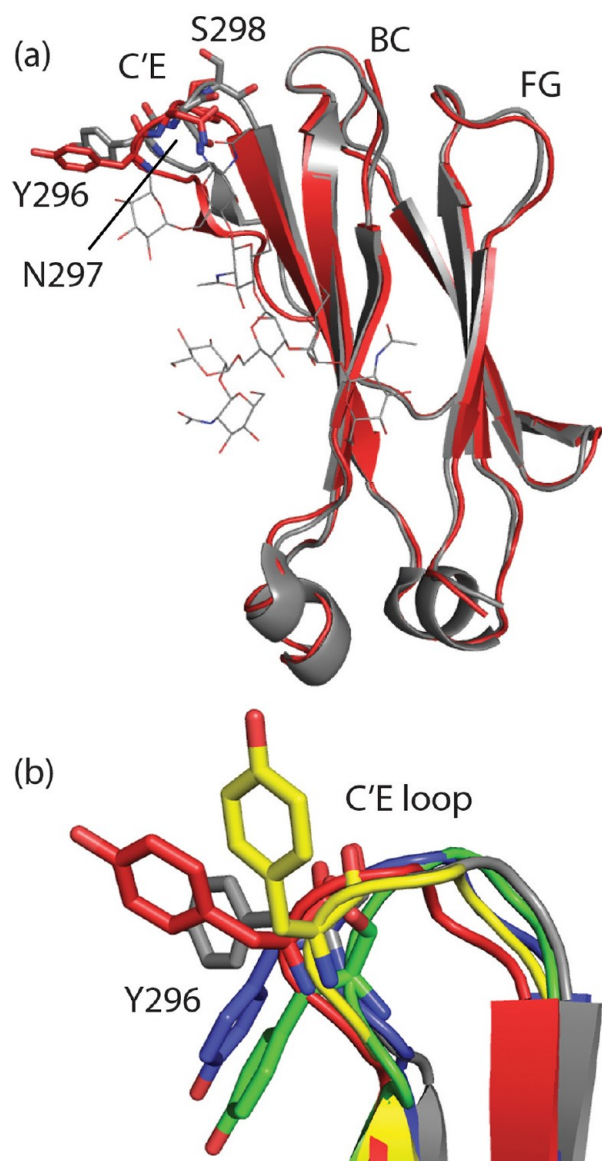
**Table 1. Data Collection and Refinement Statistics**

	Data Collection
space group	P1
	Cell Dimensions
<i>a</i> , <i>b</i> , <i>c</i> (Å)	98.2, 100.5, 116.9
$\alpha$ , $\beta$ , $\gamma$ (deg)	65.7, 65.8, 77.8
resolution (Å)	50–3.13 (3.26–3.13) <sup>a</sup>
$R_{\text{sym}}$ (%)	6.1 (34.7) <sup>a</sup>
<i>I</i> / $\sigmaI$	12.6 (2.8) <sup>a</sup>
completeness (%)	98.2 (97.1) <sup>a</sup>
redundancy	1.9 (1.9) <sup>a</sup>
	Refinement
resolution (Å)	45–3.13 (3.24–3.13) <sup>a</sup>
no. reflections	17958 (925) <sup>a</sup>
$R_{\text{cryst}}/R_{\text{free}}$ (%)	26.2/32.3 (35.1/38.3) <sup>a</sup>
	No. Atoms
protein	6640
Water	22
Wilson <i>B</i> -factor	87.2
	rms Deviations
bond lengths (Å)	0.014
bond angles (deg)	1.46
PDB code	3S7G

<sup>a</sup>Values in parentheses correspond to the highest resolution shell.

well-defined and provided unambiguous interpretation of most coordinates, with the exception of small regions of the C'E, BC, and FG loops in the upper C<sub>H</sub>2 domain of chains B and D. The high temperature factors and sparse electron density in the upper C<sub>H</sub>2 loops of B and D may be due to tight packing interactions with symmetry related Fcs within the crystal lattice.

**C'E Loop Orientation.** The orientation of the C'E loop (which harbors the Fc glycosylation site at N297) is critical for effector FcγR binding.<sup>14,24</sup> Alteration in the glycan composition<sup>14,25</sup> has been reported to cause perturbations in this loop that drastically reduce effector FcγR binding.<sup>26,27</sup> The C<sub>H</sub>2 domain of Chain A was compared with the C<sub>H</sub>2 domain of a recently solved fully glycosylated Fc (PDB ID: 3AVE)<sup>25,28</sup> revealing minor backbone deviation from the glycosylated structure in the C'E loop starting at P291 to approximately T299 (Figure 2a). The most pronounced differences are at Y296 and S298 (Figure 2a). Electron density for most of the Y296 and Q295 side chains was absent at 2 $\sigma$  density ( $2F_o - F_c$ ).



**Figure 2.** Comparison of C'E loops of glycosylated and aglycosylated  $C_{H2}$  domains. (a) The A chain  $C_{H2}$  domain of the glycosylated Fc 3AVE (gray) is aligned with the A chain of our aglycosylated  $C_{H2}$  domain (3S7G, red). Differences in the C'E loop orientation can be seen in the ribbon diagram. Y296, N297, and S298 orientations at the apex of the C'E loop differ significantly between glycosylated and aglycosylated  $C_{H2}$  domains. (b) The C'E loops of glycosylated 3AVE (gray) are shown aligned with three previously solved  $C_{H2}$  domains lacking glycosylation, 2DJ9 (yellow), 3DNK (blue), 3S7G A chain (red), and 3S7G B chain (green). Y296 side chain orientations differ significantly from the glycosylated structure and each other.

Comparison of the Y296 orientation of our structure with an aglycosylated human  $C_{H2}$  domain alone<sup>29</sup> and with an enzymatically deglycosylated Fc structure (PDB ID: 3DNK) revealed different Y296 side chain orientations relative to each other and relative to glycosylated Fc structures (Figure 2b). The dramatic variability in Y296 is notable in light of the importance of this residue in Fc $\gamma$ RIIIa and Fc $\gamma$ RIIa binding.<sup>30</sup> Additionally, the human aglycosylated Fc structure displays high crystallographic temperature factors (*B*-factors) for the C'E loop, relative to the rest of the structure (Figure 1). Indeed, a more dynamic/disordered C'E loop has been proposed to be

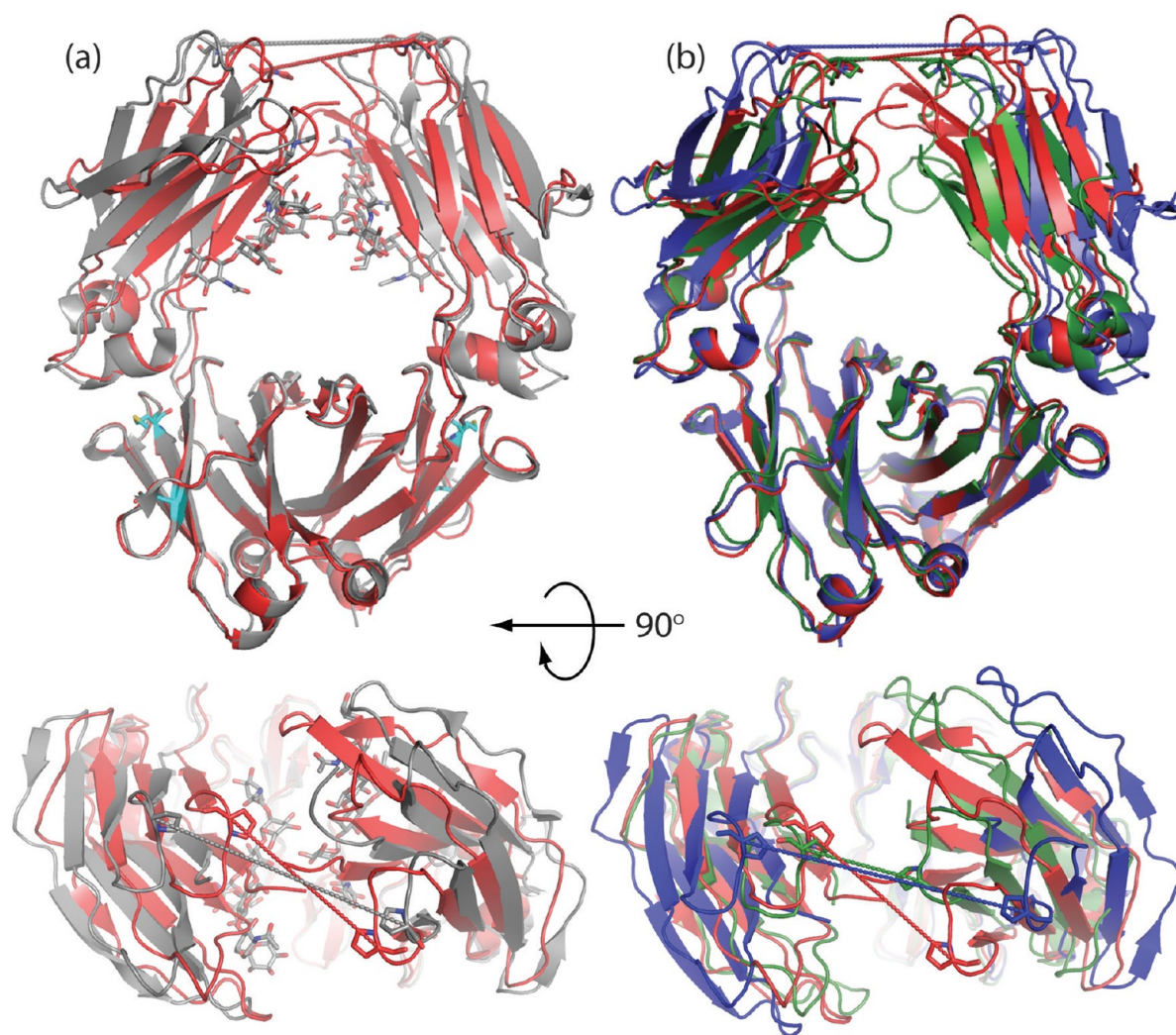
among the reasons for the lack of effector Fc $\gamma$ R binding in the absence of glycosylation.<sup>14</sup>

**Comparison with Other Fc Structures.** Superposition of the aglycosylated Fc A,B dimer with the human glycosylated Fc fragment 3AVE<sup>25,28</sup> revealed that the former adopted a more closed orientation ( $C_{H2}$  domains in closer proximity, Figure 3a). Distances between P329 (P332 in murine Fcs) in the upper  $C_{H2}$  domains had been used previously to compare the relative “openness” of the  $C_{H2}$  in Fc.<sup>14,31</sup> With P329 distances of 18.9 Å and 19.6 Å for the A,B and C,D dimers, the aglycosylated structure is significantly more closed when compared with P329 distances of fully glycosylated Fc domains (Table 2).

A further comparison with a recent structure of deglycosylated Fc (obtained by expression in mammalian cells followed by enzymatic deglycosylation) revealed a much more complex picture (Figure 3b, Table 2). Whereas the murine deglycosylated Fc (PDB ID: 3HKF) was reported to adopt a “super closed” state with the  $C_{\gamma 2}$  domains in very close proximity to one another (13.5 Å closer than in the glycosylated Fc 3AVE),<sup>15</sup> in a human deglycosylated Fc (PDB ID: 3DNK) the  $C_{H2}$  domains assume a more open structure and are farther apart relative to many glycosylated Fc domains (Table 2). The Pro329 distance in that structure is in fact 9 Å greater compared to *E. coli* purified, aglycosylated Fc A,B dimer. It should be noted that the human deglycosylated Fc differs from the human aglycosylated Fc reported here by only 3 conservative amino acid changes, one of which, N297D, is due to the action of the deglycosylase PNGase F.

**Small Angle X-ray Scattering.** The fairly dramatic difference in  $C_{H2}$  domain proximity in the three Fc structures devoid of glycan may be due to artifacts induced by crystal packing effects. Indeed, high salt conditions during crystallization have been observed to alter Fc orientation in the crystal state.<sup>14</sup> To gain a better insight into the conformation of the Fc domain in solution we employed small-angle X-ray scattering (SAXS). SAXS allows for accurate and precise measurement of a protein's radius of gyration ( $R_g$ ) and has been used previously to differentiate between open or closed conformations in solution for numerous proteins.<sup>32–34</sup> In addition to glycosylated Fc and *E. coli* expressed aglycosylated Fc, we also analyzed the shape parameters of the aglycosylated Fc5 mutant (E382V/M428I), which confers selective binding to Fc $\gamma$ RI and mediates dendritic cell activation and ADCC.<sup>20</sup>

Scattering curves of serially diluted Fc domains were extrapolated to zero concentration to account for concentration effects (Figure 4a). All three proteins exhibited Kratsky plots typical of well folded, nonaggregated samples (data not shown). As expected, scattering curves were quite similar with minor differences between the three samples visible in the higher resolution regions ( $s < 0.15$ , Figure 4a). Radii of gyration ( $R_g$ ) were determined from zero concentration extrapolations of both short (0.5 s) and long (5 s) exposure data from the slope of Guinier plots.<sup>35</sup> Small but significant difference in  $R_g$  values of glycosylated Fc, aglycosylated Fc, and the aglycosylated Fc5 mutant were observed (Table 3). Notably, the glycosylated Fc displayed an  $R_g$  of  $27.4 \pm 0.1$  Å compared to an  $R_g$  of  $28.3 \pm 0.1$  Å for aglycosylated Fc. This finding suggests that in solution the absence of glycosylation induces a more open conformation and not the “closed” form suggested by most of the crystallographic data, including ours.<sup>14,15</sup> Interestingly, the  $R_g$  of aglycosylated Fc5 ( $27.9 \pm 0.1$  Å) was intermediate to those of the glycosylated and aglycosylated Fc forms.



**Figure 3.** Comparison of overall opening/closing of  $C_H2$  domains among glycosylated and deglycosylated structures. (a) Glycosylated Fc 3AVE (gray) is compared with the A,B dimer of aglycosylated 3S7G (red). Pro 329 distances (measured from  $C\alpha$ s) are shown as dotted lines. P329 distances for 3AVE and 3S7G are 25.1 Å and 18.5 Å, respectively. E382 and M428 are represented as sticks (cyan) to highlight the location of the Fc5 mutation. (b) The A,B dimer of aglycosylated 3S7G (red) is compared with deglycosylated human Fc, 3DNK (blue), and the deglycosylated mouse Fc, 3HKF (green). P329 distances for 3DNK and 3HKF are 27.6 Å and 11.6 Å, respectively.

**Table 2. Proline 329 Distances for Fc Structures with and without N297 Glycans; Structures Lacking the N297 Glycan Are in Italics**

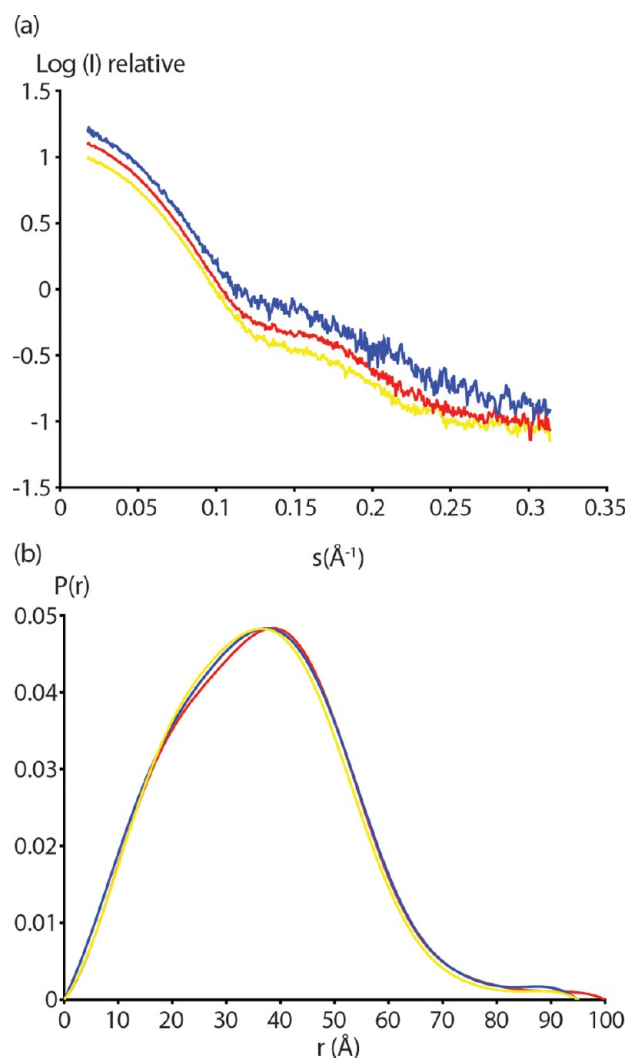
PDB ID	chains	space group	resolution (Å)	P329 distance (Å)	reference
3S7G	A,B	P1	3.1	18.9	this study
3S7G	C,D	P1	3.1	19.6	this study
3DNK	A,B	<i>P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub></i>	2.8	27.6	unpublished
3HKF	A	<i>P6<sub>1</sub>22</i>	2.5	11.6 <sup>a</sup>	15
3AVE	A,B	<i>P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub></i>	2.0	25.1	25, 28
2DTS	A,B	<i>P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub></i>	2.2	24.2	25
1H3Y	A,B	<i>P6<sub>1</sub>22</i>	4.1	29.6	14
1H3W	M	<i>C222<sub>1</sub></i>	2.85	33.8	14
1H3X	A,B	<i>P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub></i>	2.4	22.6	14
1H3V	A,B	<i>P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub></i>	2.4	26.9	14
1FC1	A,B	<i>P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub></i>	2.9	26.8	48
1HZH	A,B	<i>H32</i>	2.7	23.5	49

<sup>a</sup>For the murine Fc (2HKF) Pro332 distances are measured.

The interparticle distance distribution functions or pairwise distributions,  $P(r)$  derived from the scattering curves were also compared (Figure 4b). The maximum diameter for aglycosylated Fc was approximated at 100 Å, whereas the aglycosylated Fc5 and glycosylated Fc had a maximum diameter of 95 Å. The  $R_g$  values derived from  $P(r)$  curves were consistent with the calculated values from the Guinier analysis (Table 3). Minor differences in between the three  $P(r)$  curves can be seen in the 20–35 Å regions with the aglycosylated Fc5 curve falling in between the glycosylated Fc and aglycosylated Fc.

## DISCUSSION

In this study, we describe the structure of a human, aglycosylated, *E. coli* expressed Fc domain determined by X-ray crystallography. The major structural differences between fully glycosylated Fc domains capable of binding to immune effector receptors and the aglycosylated structure presented herein are a “closing” of the  $C_H2$  domains and disorder within the C'E loop containing N297 (the glycan attachment point on the  $C_H2$  domain).



**Figure 4.** SAXS analysis of glycosylated and aglycosylated Fcs. (a) Scattering profiles from 0.5 s exposure are shown for glycosylated Fc (yellow) aglycosylated Fc (red) and aglycosylated Fc5 (blue). These profiles were obtained by extrapolating curves at different concentrations to the zero concentration. The logarithm of scattering intensity is shown as a function of the reciprocal vector ( $S$ ). (b) The distance distribution functions ( $P(r)$ ) of glycosylated Fc (yellow), aglycosylated Fc (red), and aglycosylated Fc5 (blue) are shown. Curves were calculated from the SAXS scattering data in panel a.

**Table 3. Observed  $R_g$  Values for Aglycosylated and Glycosylated Fcs (in Å)**

	$R_g$ Guinier		$R_g$ $P(r)$
	(0.5 s exposure)	(5 s exposure)	
aglycosylated Fc (wt)	28.3 ± 0.1	28.4 ± 0.1	28.2 ± 0.1
aglycosylated Fc5	27.9 ± 0.1	28.0 ± 0.2	28.0 ± 0.1
glycosylated Fc (wt)	27.4 ± 0.1	27.2 ± 0.1	27.5 ± 0.1

C'E loop flexibility has previously been reported in glycan truncated Fc structures as well as in a deglycosylated murine Fc and a human aglycosylated  $C_{H2}$  domain.<sup>14,15,29</sup> The normally rigid glycosylated C'E loop is known to be crucial to certain Fc $\gamma$ R binding interactions.<sup>36</sup> The lack of glycan stabilization of the C'E loop noted in our structure and others is likely at least partially responsible for the lack of effector binding in Fc domains lacking glycans. Recent crystallographic data also

indicated that carbohydrates on Fc $\gamma$ RIII can productively interact with Fc N297 carbohydrates in the absence of fucosylation.<sup>37</sup> Fc domains lacking N297 glycosylation would lose any beneficial carbohydrate-carbohydrate interactions for Fc $\gamma$ RIII binding as well.

In addition to changes in the C'E loop, a more closed orientation of  $C_{H2}$  domains is noticeable in both aglycosylated Fc dimers present in our structure. A progressive closure of the  $C_{H2}$  domains has been noted in crystal structures of Fcs with enzymatically truncated<sup>14</sup> and fully removed glycans.<sup>15</sup> This collapse of the  $C_{H2}$  domains has also been suggested to be at least partially responsible for the loss of effector affinity.<sup>15</sup> A deglycosylated Fc structure (PDB ID: 3DNK) has also been deposited without a closed  $C_{H2}$  domain. Indeed, 3DNK has a more open orientation than even some glycosylated Fc structures (Table 2). This deglycosylated yet more open structure counters the notion that removing N297 glycans leaves a closed, collapsed Fc. Taken as a whole, the crystallographic evidence suggests that Fcs lacking glycans can adopt a number of possible orientations, and the observed orientation in crystals may be highly dependent on the crystal packing environment and therefore might not reflect the state of these molecules in solution. Interestingly, differences in the degree of "openness" between fully glycosylated Fc structures have also been noted,<sup>31</sup> although not nearly to the extent seen in Fc structures lacking glycans (Figure 3, Table 2). We speculate that part of the role of the N297 glycan is to limit the dynamic nature of the  $C_{H2}$  domains and restrict range of movement of the domains.

To reconcile the disparate crystallographic data on the "openness" of the aglycosylated Fc domain, we chose to examine structural parameters in solution *via* SAXS. SAXS data indicated surprisingly that *E. coli* produced aglycosylated Fc domains actually had a larger  $R_g$  compared to glycosylated human Fc, thus suggesting that aglycosylated Fc domains have a more open  $C_{H2}$  orientation in solution. SAXS scattering curves describe an average of the possible conformations a protein adopts in solution. The collapsed  $C_{H2}$  orientations seen in X-ray structures may be one of multiple forms that Fc domains lacking glycans are able to adopt; however, our SAXS data suggest that in solution they commonly adopt a conformation with a higher  $R_g$  compared to glycosylated Fc domains.

Interestingly, the aglycosylated Fc mutant Fc5 (E382V/M428I) had a  $R_g$  value 0.4 Å smaller than that of wildtype aglycosylated Fc (Table 3). This smaller  $R_g$  may be indicative of a structural change caused by the mutations in  $C_{H3}$  that increases binding to Fc $\gamma$ RI at the apex of the  $C_{H2}$  domain. We suspected that the  $C_{H2}$ – $C_{H3}$  region of aglycosylated Fcs may be fertile ground for allosterically modifying distal effector functions.<sup>19,20</sup>

## METHODS

**Expression and Purification of Aglycosylated Fc Proteins.** *E. coli* Jude-1 (F' [Tn10(Tet<sup>r</sup>) proAB<sup>+</sup> lacI<sup>r</sup>  $\Delta$ (lacZ)M15] *mcrA*  $\Delta$ (*mrr-hsdRMS-mcrBC*)  $\phi$ 80dlacZ $\Delta$ M15  $\Delta$ lacX74 *deoR* *recA1* *araD139*  $\Delta$ (*ara* *leu*)7697 *galU* *galK* *rpsL* *endA1* *nupG*) cells harboring pTrc99A-DsbA-Fc<sup>20</sup> encoding either wild-type or Fc5 were cultured in 4 L flasks with 2.5 L working volume of Terrific Broth (TB) media at 37 °C. The shake flasks were transferred to 30 °C, 1 mM IPTG was added, and cells (OD<sub>600</sub> approximately 0.6) were grown overnight (14 h). The cells were then harvested, and the supernatant was separated from cells by centrifugation at 9,000 rpm for 30 min. The culture supernatant was then filtered through a 0.45  $\mu$ m filter. One milliliter of

Immobilized Protein A agarose (Pierce Biotechnology) was added to the filtered supernatant and incubated overnight at 4 °C with gentle stirring. The immobilized Protein A agarose was then collected in a column and washed with 50 mL of PBS (pH 7.2). The column was then eluted using 0.1 M glycine (pH 2.7) and neutralized immediately with 1 M Tris (pH 8.0). Fc-containing fractions were concentrated by ultrafiltration (10 kDa  $M_w$  cutoff) and purified by gel filtration on a Superdex G75 column (Amersham Pharmacia). Aglycosylated Fc, FcS, and glycosylated Fc (Bethyl Laboratories) were then concentrated by ultrafiltration (10 kDa  $M_w$  cutoff), and the buffer was exchanged to 20 mM Tris (pH 8) with 5% glycerol. All Fc domains were confirmed to be dimers in solution by gel filtration.

**X-ray Crystallography.** Crystals of aglycosylated Fc were obtained in sitting drops using 15 mg mL<sup>-1</sup> Fc added 1:1 to a mother liquor of 10% PEG 1000, 20% PEG 6000, 0.1 M magnesium chloride, and 0.2 M Tris HCL, pH 7.0. Cryo-cooling in liquid nitrogen was achieved using the mother liquor plus 20% glycerol as a cryoprotectant. A 3.1 Å resolution data set was collected from Beamline 8.3.1 at the Advanced Light Source (Lawrence Berkeley National Laboratories). Diffraction data were scaled and integrated using HKL2000.<sup>38</sup> Molecular replacement was successfully performed using PHASER<sup>39,40</sup> with the C<sub>H</sub>2 and C<sub>H</sub>3 domains from PDB 3DNK as the search model. Two dimers were found in the asymmetric unit using PHASER. The model was refined using Refmac<sup>41</sup> in the CCP4i suite.<sup>42</sup> Noncrystallographic symmetry (NCS) restraints<sup>43</sup> were used throughout refinement with C<sub>H</sub>2 and C<sub>H</sub>3 domains of chains A–D linked with the exception of residues 335–345 (the region connecting C<sub>H</sub>2 and C<sub>H</sub>3) and the C'E loops. A and C chains and B and D chains of the C'E loop were linked by NCS due to tight crystal packing in the C'E loops of chains B and D. No electron density was detected for N-terminal residues 221–235 (hinge residues) and C terminal residues 445–447; these residues are not in the models. Figures were generated with PyMOL.<sup>44</sup>

**Small Angle X-ray Scattering.** Prior to the SAXS experiments, all Fc domains were dialyzed into a 20 mM Tris (pH 8) buffer with 5% glycerol as a cryoprotectant. SAXS data were collected at the SIBYLS beamline of the Advanced Light Source (Lawrence Berkeley National Laboratories). SAXS data were collected on 3 serial dilutions of each sample preparation at 5, 2.5, and 1.25 mg mL<sup>-1</sup>. All data were collected with 1 Å X-rays. Three exposures were taken for each sample, first a short exposure (0.5 s) and then a long exposure of 5 s, followed by an additional 0.5 s exposure to check for radiation damage. The sample cell was washed between protein solutions using a mild detergent soak followed by 3 rinses with buffer solution. Data for buffer alone was also collected before and after each protein sample and subtracted from the sample curve.

The radius of gyration ( $R_g$ ), which is defined as the root mean squared (rms) distance of all atoms from their common center of mass, was derived using Guinier analysis<sup>35</sup> in regions where  $QR_g < 1.3$  using the program PRIMUS.<sup>45,46</sup> Pair distribution functions were generated using the program GNOM.<sup>47</sup> The maximum diameter of the particle ( $D_{max}$ ) was adjusted in 5 Å increments in GNOM to maximize the goodness-of-fit parameter.

## ■ ASSOCIATED CONTENT

### Accession Codes

The coordinates of the aglycosylated human IgG1 Fc have been deposited in the Protein Data Bank with the accession number 3S7G.

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### Author Contributions

M.J.B. and G.G. designed research; M.J.B., T.H.K., S.T.J., and A.F.M. performed research and generated reagents; M.J.B. and G.G. analyzed data; M.J.B. and G.G. wrote the paper.

## Notes

The authors declare no competing financial interest.

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## ■ REFERENCES

- (1) Nimmerjahn, F., and Ravetch, J. (2007) Antibodies, Fc receptors and cancer. *Curr. Opin. Immunol.* 19, 239–245.
- (2) Nimmerjahn, F., and Ravetch, J. (2008) Fc receptors as regulators of immune responses. *Nat. Rev. Immunol.* 8, 34–47.
- (3) Jefferis, R., Lund, J., and Pound, J. D. (1998) IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. *Immunol. Rev.* 163, 59–76.
- (4) Jefferis, R. (2009) Glycoforms of human IgG in health and disease. *Trends Glycosci. Glycotechnol.* 21, 105–117.
- (5) Nesspor, T. C., Raju, T. S., Chin, C.-N., Vafa, O., and Brezski, R. J. (2012) Avidity confers FcγR binding and immune effector function to aglycosylated immunoglobulin G1. *J. Mol. Recognit.* 25, 147–154.
- (6) Tao, M. H., and Morrison, S. L. (1989) Studies of aglycosylated chimeric mouse-human IgG - Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J. Immunol.* 143, 2595–2601.
- (7) Walker, M. R., Lund, J., Thompson, K. M., and Jefferis, R. (1989) Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing FcγRI and or FcγRII receptors. *Biochem. J.* 259, 347–353.
- (8) Beck, A., Cochet, O., and Wurch, T. (2010) GlycoFit's technology to control the glycosylation of recombinant therapeutic proteins. *Expert Opin. Drug Discovery* 5, 95–111.
- (9) Jefferis, R. (2009) Glycosylation as a strategy to improve antibody-based therapeutics. *Nat. Rev. Drug Discovery* 8, 226–234.
- (10) Niwa, R., Sakurada, M., Kobayashi, Y., Uehara, A., Matsushima, K., Ueda, R., Nakamura, K., and Shitara, K. (2005) Enhanced natural killer cell binding and activation by low-fucose IgG1 antibody results in potent antibody-dependent cellular cytotoxicity induction at lower antigen density. *Clin. Cancer Res.* 11, 2327–2336.
- (11) Okazaki, A., Shoji-Hosaka, E., Nakamura, K., Wakitani, M., Uchida, K., Kakita, S., Tsumoto, K., Kumagai, I., and Shitara, K. (2004) Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcγRIIIa. *J. Mol. Biol.* 336, 1239–1249.
- (12) Shields, R. L., Lai, J., Keck, R., O'Connell, L. Y., Hong, K., Meng, Y. G., Weikert, S. H. A., and Presta, L. G. (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and antibody-dependent cellular toxicity. *J. Biol. Chem.* 277, 26733–26740.
- (13) Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N., and Shitara, K. (2003) The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human

- IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J. Biol. Chem.* 278, 3466–3473.
- (14) Krapp, S., Mimura, Y., Jefferis, R., Huber, R., and Sondermann, P. (2003) Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. *J. Mol. Biol.* 325, 979–989.
- (15) Feige, M. J., Nath, S., Catharino, S. R., Weinfurter, D., Steinbacher, S., and Buchner, J. (2009) Structure of the murine unglycosylated IgG1 Fc fragment. *J. Mol. Biol.* 391, 599–608.
- (16) Jefferis, R. (2009) Aglycosylated antibodies and the methods of making and using them: WO2008030564. *Expert Opin. Ther. Pat.* 19, 101–105.
- (17) Mazor, Y., Van Blarcom, T., Mabry, R., Iverson, B., and Georgiou, G. (2007) Isolation of engineered, full-length antibodies from libraries expressed in *Escherichia coli*. *Nat. Biotechnol.* 25, 563–565.
- (18) Simmons, L., Reilly, D., Klimowski, L., Shantha Raju, T., Meng, G., Sims, P., Hong, K., Shields, R., Damico, L., and Rancatore, P. (2002) Expression of full-length immunoglobulins in *Escherichia coli*: rapid and efficient production of aglycosylated antibodies. *J. Immunol. Methods* 263, 133–147.
- (19) Jung, S. T., Kang, T. H., Kelton, W., and Georgiou, G. (2011) Bypassing glycosylation: engineering aglycosylated full-length IgG antibodies for human therapy. *Curr. Opin. Biotechnol.* 22, 858–67.
- (20) Jung, S. T., Reddy, S. T., Kang, T. H., Borrok, M. J., Sandlie, I., Tucker, P. W., and Georgiou, G. (2010) Aglycosylated IgG variants expressed in bacteria that selectively bind Fc gamma RI potentiate tumor cell killing by monocyte-dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 107, 604–609.
- (21) Sazinsky, S. L., Ott, R. G., Silver, N. W., Tidor, B., Ravetch, J. V., and Wittrup, K. D. (2008) Aglycosylated immunoglobulin G1 variants productively engage activating Fc receptors. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20167–20172.
- (22) Lu, J. H., Ellsworth, J. L., Hamacher, N., Oak, S. W., and Sun, P. D. (2011) Crystal Structure of Fc gamma Receptor I and Its Implication in High Affinity gamma-Immunoglobulin Binding. *J. Biol. Chem.* 286, 40608–40613.
- (23) Shields, R. L., Namenuk, A. K., Hong, K., Meng, Y. G., Rae, J., Briggs, J., Xie, D., Lai, J., Stadlen, A., Li, B., Fox, J. A., and Presta, L. G. (2001) High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J. Biol. Chem.* 276, 6591–6604.
- (24) Radaev, S., and Sun, P. (2002) Recognition of immunoglobulins by Fc gamma receptors. *Mol. Immunol.* 38, 1073–1083.
- (25) Matsumiya, S., Yamaguchi, Y., Saito, J., Nagano, M., Sasakawa, H., Otaki, S., Satoh, M., Shitara, K., and Kato, K. (2007) Structural comparison of fucosylated and nonfucosylated Fc fragments of human immunoglobulin G1. *J. Mol. Biol.* 368, 767–779.
- (26) Mimura, Y., Church, S., Ghirlando, R., Ashton, P. R., Dong, S., Goodall, M., Lund, J., and Jefferis, R. (2000) The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms. *Mol. Immunol.* 37, 697–706.
- (27) Mimura, Y., Sondermann, P., Ghirlando, R., Lund, J., Young, S. P., Goodall, M., and Jefferis, R. (2001) Role of oligosaccharide residues of IgG1-Fc in Fc gamma RIIb binding. *J. Biol. Chem.* 276, 45539–45547.
- (28) Matsumiya, S., Yamaguchi, Y., Saito, J., Nagano, M., Sasakawa, H., Otaki, S., Satoh, M., Shitara, K., and Kato, K. (2011) Corrigendum to "Structural Comparison of Fucosylated and Nonfucosylated Fc Fragments of Human Immunoglobulin G1" (vol 386, pg 767, 2007). *J. Mol. Biol.* 408, 1001–1001.
- (29) Prabakaran, P., Vu, B. K., Gan, J. H., Feng, Y., Dimitrov, D. S., and Ji, X. H. (2008) Structure of an isolated unglycosylated antibody C(H)2 domain. *Acta Crystallogr. D* 64, 1062–1067.
- (30) Kato, K., Sautes-Fridman, C., Yamada, W., Kobayashi, K., Uchiyama, S., Kim, H., Enokizono, J., Galinha, A., Kobayashi, Y., Fridman, W. H., Arata, Y., and Shimada, I. (2000) Structural basis of the interaction between IgG and Fc gamma receptors. *J. Mol. Biol.* 295, 213–224.
- (31) Oganessian, V., Damschroder, M. M., Leach, W., Wu, H., and Dall'Acqua, W. F. (2008) Structural characterization of a mutated, ADCC-enhanced human Fc fragment. *Mol. Immunol.* 45, 1872–1882.
- (32) Borrok, M. J., Zhu, Y. M., Forest, K. T., and Kiessling, L. L. (2009) Structure-based design of a periplasmic binding protein antagonist that prevents domain closure. *ACS Chem. Biol.* 4, 447–456.
- (33) Komeiji, Y., Ueno, Y., and Uebayasi, M. (2002) Molecular dynamics simulations revealed Ca<sup>2+</sup>-dependent conformational change of calmodulin. *FEBS Lett.* 521, 133–139.
- (34) Shilton, B., Flocco, M., Nilsson, M., and Mowbray, S. (1996) Conformational changes of three periplasmic receptors for bacterial chemotaxis and transport: the maltose-, glucose/galactose- and ribose-binding proteins. *J. Mol. Biol.* 264, 350–363.
- (35) Guinier, A., and Fournet, G. (Eds.) (1955) *Small-Angle Scattering of X-rays*, John Wiley and Sons, New York.
- (36) Sondermann, P., Huber, R., Oosthuizen, V., and Jacob, U. (2000) The 3.2-angstrom crystal structure of the human IgG1 Fc fragment-Fc gamma RIII complex. *Nature* 406, 267–273.
- (37) Ferrara, C., Grau, S., Jager, C., Sondermann, P., Brunker, P., Waldhauer, I., Hennig, M., Ruf, A., Rufer, A. C., Stihle, M., Umama, P., and Benz, J. (2011) Unique carbohydrate-carbohydrate interactions are required for high affinity binding between Fc gamma RIII and antibodies lacking core fucose. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12669–12674.
- (38) Otwinowski, Z., and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* 276, 307–326.
- (39) McCoy, A., Grosse-Kunstleve, R., Storoni, L., and Read, R. (2005) Likelihood-enhanced fast translation functions. *Acta Crystallogr. D* 61, 458–464.
- (40) Storoni, L., McCoy, A., and Read, R. (2004) Likelihood-enhanced fast rotation functions. *Acta Crystallogr. D* 60, 432–438.
- (41) Winn, M., Murshudov, G., and Papiz, M. (2003) Macromolecular TLS refinement in REFMAC at moderate resolutions. *Method Enzymol.* 374, 300–321.
- (42) Winn, M. (2002) An overview of the CCP4 project in protein crystallography: an example of a collaborative project. *J. Synchrotron Radiat.* 10, 23–25.
- (43) Kleywegt, G. (1996) Use of non-crystallographic symmetry in protein structure refinement. *Acta Crystallogr. D* 52, 842–857.
- (44) DeLano, W. (2002) *The PyMOL Molecular Graphics System*, DeLano Scientific, Palo Alto, CA, USA; <http://www.pymol.org>.
- (45) Konarev, P., Petoukhov, M., Volkov, V., and Svergun, D. (2006) ATSAS 2.1, a program package for small-angle scattering data analysis. *J. Appl. Crystallogr.* 39, 277–286.
- (46) Konarev, P., Volkov, V., Sokolova, A., Koch, M., and Svergun, D. (2003) PRIMUS: a Windows PC-based system for small-angle scattering data analysis. *J. Appl. Crystallogr.* 36, 1277–1282.
- (47) Svergun, D. I. (1999) Restoring low resolution structure of biological macromolecules from solution scattering using simulated annealing. *Biophys. J.* 76, 2879–2886.
- (48) Deisenhofer, J. (1981) Crystallographic refinement and atomic models of a human Fc Fragment and its complex with fragment-B of Protein-A from *Staphylococcus aureus* at 2.9 Å and 2.8 Å resolution. *Biochem.* 20, 2361–2370.
- (49) Saphire, E. O., Parren, P., Pantophlet, R., Zwick, M. B., Morris, G. M., Rudd, P. M., Dwek, R. A., Stanfield, R. L., Burton, D. R., and Wilson, I. A. (2001) Crystal structure of a neutralizing human IgG against HIV-1: A template for vaccine design. *Science* 293, 1155–1159.