REVIEW

Biophysical characterization of dynamic structures of immunoglobulin G

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Abstract

Immunoglobulin G (IgG) is a major antibody and functions as a hub linking specific antigen binding and recruitment of effector molecules typified by Fcγ receptors (FcγRs). These activities are associated primarily with interactions involving its Fab and Fc sites, respectively. An IgG molecule is characterized by a multiple domain modular structure with conserved N-glycosylation in Fc. The molecule displays significant freedom in internal motion on various spatiotemporal scales. The consequent conformational flexibility and plasticity of IgG glycoproteins are functionally significant and potentially important factors for design and engineering of antibodies with enhanced functionality. In this article, experimental and computational approaches are outlined for characterizing the conformational dynamics of IgG molecules in solution. In particular, the importance of integration of these approaches is highlighted, as illustrated by dynamic intramolecular interactions between the pair of N-glycans and their proximal amino acid residues in Fc. These interactions can critically affect effector functions mediated by human IgG1 and FcγRIII. Further improvements in individual biophysical techniques and their integration will advance understanding of dynamic behaviors of antibodies in physiological and pathological conditions. Such understanding will provide opportunities for engineering antibodies through controlling allosteric networks in IgG molecules.

Keywords Immunoglobulin G · Antibody · Fcγ receptor · N-glycan · Molecular dynamics simulation · Solution scattering · X-ray crystallography · Nuclear magnetic resonance spectroscopy · Core fucosylation · Dynamic conformational ensemble

Introduction

Immunoglobulin G (IgG) is a glycoprotein composed of multiple homologous domains (the so-called Ig domains) and plays key roles as an immune system antibody (Chiu et al. 2019) (Fig. 1). This glycoprotein consists of two identical light chains, each divided into V_L and C_L domains, and two identical heavy chains, each containing V_H, C_H1, C_H2, and C_H3 domains. C_H1 and C_H2 domains are connected by a protease-susceptible hinge segment. Cleavage of this segment

gives rise to two Fab fragments constituted by V_L, V_H, C_L, and C_H1 domains and one Fc fragment constituted by two C_H2 and two C_H3.

Major functions of IgG are recognition of antigens on surfaces of invading viruses and bacteria and recruitment of effector molecules, such as complement component C1 and Fcγ receptors (FcγRs), for elimination of such pathogens. Thus, IgG serves as a hub that links these two functions.

V_H and V_L domains are structurally variable and are responsible for antigen recognition. The remaining domains are much less divergent but are classified into several isotypes. The constant region of the IgG heavy chain defines subclasses—IgG1–4 in humans. V_H and V_L domains each display three hypervariable loops that are directly involved in specific antigen binding and are thus often referred to as complementarity-determining regions (CDRs). Each C_H2 domain of Fc homodimer possesses one conserved N-glycosylation site (Asn297) A biantennary complex-type oligosaccharide is expressed at this site, with microheterogeneity resulting from nonreducing terminal fucose (Fuc), galactose (Gal), bisecting N-acetylglucosamine (GlcNAc), and sialic

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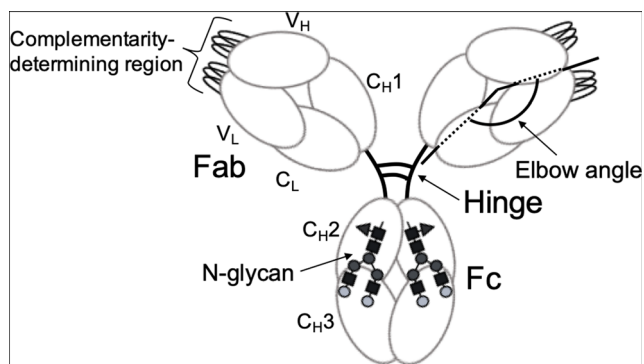
Fig. 1 Schematic drawing of IgG. An IgG molecule is characterized by a multiple domain modular structure with conserved N-glycosylation in Fc and significant freedom for internal motion

acid residues (Yamaguchi et al. 2007). This N-glycosylation is essential for interactions with effector molecules, which are particularly affected by terminal structures of N-glycans.

Currently, IgGs are widely used for detection, quantification, and characterization of biological and pathological molecules and as biopharmaceuticals that target diseases, including cancers. A variety of engineered IgG antibodies and their derivatives have been developed and used for diagnostic and therapeutic purposes (Chiu et al. 2019). The structure of IgG is characterized by considerable conformational flexibility and plasticity, which are supposed to be of relevance to antigen binding and interactions with the effectors (Jay et al. 2018; Yang et al. 2017). An IgG molecule possesses hierarchical degrees of freedom in internal motion across various spatio-temporal scales. This conformational dynamic of IgG is critical for design and engineering of recombinant antibodies with enhanced functionality for interactions with antigens and effector molecules.

In this review, dynamic views of IgG structures are outlined, highlighting the importance of integration of experimental and computational approaches.

Experimental approaches for investigating IgG conformational dynamics

Early X-ray crystallographic studies of monoclonal IgGs and their light chains derived from multiple myeloma patients revealed their modular structures—Ig domains exhibiting longitudinal and transverse interactions within Fab portions (Schiffer et al. 1973; Edmundson et al. 1975). However, crystal structures provided no interpretable electron density for the Fc portion (Colman et al. 1976; Marquart et al. 1980). In contrast, crystallographic data of naturally occurring IgG mutants that lack hinge segments were able to visualize both Fab and Fc (Silverton et al. 1977; Rajan et al. 1983). These findings suggested that internal motion of IgG molecules is attributable to the flexible nature of the hinge region. Subsequently,

crystal structures were reported for an Fc fragment cleaved from human IgG1 that displayed a pair of oligosaccharides packed between the two C_H2 domains (Deisenhofer 1981). Three-dimensional structures of intact IgG molecule closely packed in the crystal lattice were eventually visualized in 1992 (Harris et al. 1992). Concurrently, crystallographic data were accumulated for Fab fragments complexed with their specific antigens (or haptens), followed by reports of crystal structures of Fc fragments bound to their physiological and pathological binding proteins (Padlan 1994; Yamaguchi et al. 2007).

These studies underscore the significant freedom of internal motion in Fab and Fc antibody fragments. Conformational changes in CDR loops in variable domains of Fab exhibited conformational differences between free and antigen-bound forms (Padlan 1994; Poljak 1975). In particular, the third CDR in the V_H domain often exhibited conformational polymorphism even among crystal structures in the absence of antigen (Nakasako et al. 2005; Fernández-Quintero et al. 2018). This suggests that dynamic properties of antigen-binding loops for induced-fit and conformational selection mechanisms underlie antigen recognition. Crystallographic data also demonstrate orientation adjustment of V_H and V_L domains upon binding to antigen (Dunbar et al. 2013). Fab crystal structures show significant variation of the elbow angle, defined as the angle between pseudo-twofold axes for V_L to V_H and C_L to C_H1 (Stanfield et al. 2006). Cumulative crystallographic data for Fc also indicate variation in quaternary structures, which might depend on glycoforms and conformational transition of Fc on binding partners such as FcγRs (Yagi, Yanaka et al. 2018; Yamaguchi et al. 2007; Yanaka et al. 2019a). These conformational variations observed in crystal structures should be carefully interpreted because such structural snapshots are unavoidably influenced by crystal packing.

Solution structures of IgG molecules have been characterized using scattering and spectroscopic techniques. The former provides overall structure information of antibodies in solution, and the latter enables microscopic characterization of dynamic structures. Small-angle X-ray scattering (SAXS) has been used to delineate solution structures of IgG. Comparative SAXS analyses successfully revealed differences in orientation of Fab arms among mouse and human IgG subclasses, presumably dependent on their hinge structures (Gregory et al. 1987; Igarashi et al. 1990), as suggested by conventional electron microscopy (EM) and fluorescence depolarization measurements (Roux et al. 1997; Schneider et al. 1988; Tan et al. 1990; Oi et al. 1984). SAXS analysis also revealed mutational alteration of the quaternary structure of IgG-Fc. In contrast to SAXS, small-angle neutron scattering (SANS) can distinguish isotopic species, especially ^{1}H and ^{2}H by differences in neutron scattering length densities (Bernado et al. 2018). This ability enables observation of specific protein components in complexes by selective

deuteration combined with a contrast matching. This method was used to examine spatial arrangements of selectively deuterated antigenic proteins bound to the Fab arms in different mouse IgG subclasses. In particular, distances between two antigen-binding sites were estimated (Sosnick et al. 1992). Further, a recent study demonstrated the capability of SANS to detect quaternary structure deformation of IgG1-Fc by interaction with 75% deuterated FcγRIIIb in a ^{2}H$_2$O solution (Yogo et al. 2017). Considering the partial dissociation of the low-affinity complex, the SANS data were successfully interpreted based on the previously reported crystal structure of the complex.

IgG structures in solution are depicted as dynamic conformational ensembles, which are not reproduced solely based on solution scattering observations. To interpret solution scattering results, possible conformational space occupied by target proteins must be defined (Bernado et al. 2018). This definition could use computational simulations based on structural data obtained from crystal structures. Recent advances in EM image analysis make it possible to visualize conformational spaces occupied by whole IgG molecules (Jay et al. 2018). The integration of the above methods is critical to providing dynamic views of IgG molecules in solution.

In contrast to solution scattering, nuclear magnetic resonance (NMR) spectroscopy provides information on antibody conformational dynamics over a broad range of timescales at atomic resolution (Arata et al. 1994; Barb 2017). Earlier NMR studies of an anti-DANSYL Fv fragment, a heterodimer of V$_H$ and V$_L$ domains, highlighted slow conformational dynamics, at a millisecond timescale, of the CDR3 loop, a primary binding site of the hapten (Odaka et al. 1992; Takahashi et al. 1994). A recent NMR study showed that the framework region of an anti-lysozyme Fv exhibited conformational fluctuation on a sub-microsecond timescale (Yanaka et al. 2017a). Moreover, a mutation at this site suppressed the conformational fluctuations, resulting in an increased affinity for the antigen. This finding suggests a potential strategy for engineering antibodies to improve antigen-binding properties through use of mutation at a region outside of the binding site.

Dynamic structures of IgG hinge regions have been investigated by a series of NMR studies, revealing a disulfide-linked rigid core (termed *core hinge*) flanked by extremely flexible segments (Matsunaga et al. 1991; Kim et al. 1994). Cleavage of the disulfide bridge renders the core hinge flexible and impairs interactions of IgG with effector proteins. NMR data also demonstrated that the hinge and the following Fc portions remain mobile even in immune complexes where Fab arms are cross-linked by multivalent antigens (Kim 1994). This result is consistent with suggestions from crystallographic studies. The C$_H$1-proximal flexible segment (termed *upper hinge*) endows mobility to the Fab arm, and the flexible segment proximal to the C$_H$2 domain (termed *lower hinge*) is involved in interaction with FcγRs.

Antibodies undergo much slower conformational transition at timescales of seconds or longer. These transitions can be detected by hydrogen-deuterium exchange experiments using NMR spectroscopy and, more recently, mass spectrometry. These techniques can offer useful probes for examining global unfolding of Ig domains, domain-domain interactions, and ligand binding of IgG molecules (Kawata et al. 1988; Shimba et al. 1995; Yogo et al. 2019; Zhang et al. 2011).

Stable isotope labeling of proteins is essential for a detailed NMR study of dynamic structures. Because glycoproteins are not expressed in bacterial systems, various eukaryotic vehicles have been developed for producing isotopically labeled IgG glycoproteins (Yanaka et al. 2018; Kato et al. 2018b; Kato et al. 2010; Yamaguchi et al. 2017).

High-speed atomic force microscopy (HS-AFM) offers a powerful emerging tool for observing dynamic behaviors of IgG. Several studies have been reported real-time observation of the interactions of IgG molecules with antigens and effector molecules on membrane-like surfaces (Yogo et al. 2019; Yanaka et al. 2019b; Strasser et al. 2019).

Computational tools for exploring IgG conformational spaces

Computational approaches exemplified by MD and Monte Carlo simulations offer powerful tools for providing ensembles of biomolecular structures (Hansmann et al. 1996). MD simulation has been applied to IgG and its fragments to describe physical movements in solution (Frank et al. 2014; Lee and Im 2017; Yanaka et al. 2017a; Yanaka et al. 2019a). MD simulations with all atoms of glycans, proteins, and surrounding waters are a straightforward way to obtain conformational ensembles. To sufficiently cover the conformation spaces, a longer-time simulation is necessary, which is conducted often by employing parallel computing for improving conformation sampling (Hansmann 2003). Despite high computational cost, conventional all-atom MD simulation is insufficient for adequately exploring conformational spaces, especially spaces occupied by large, complicated biomacromolecules (Lane et al. 2013).

Several methods have been proposed to enhance the efficiency of conformational sampling, including metadynamics and simulated annealing (Bernardi et al. 2015). One efficient method is replica-exchange molecular dynamics simulation (Sugita and Okamoto 1999). This method has been applied to explore conformational spaces of various oligosaccharides and glycans in complexes with of IgG1-Fc and FcγRIIIa (Yagi et al. 2018b; Yagi-Utsumi et al. 2017; Kato et al. 2018a). In the latter case, protein backbone conformations were mostly constrained, as those in the initial crystal structure in order to minimize calculation costs.

A bolder approach in molecular simulation is based on a coarse-grained modeling, in which proteins are represented by pseudo-atoms approximating whole amino acid residues to reduce the degrees of freedom and thereby enable much longer simulation in comparison with all-atom simulations (Ingólfsson et al. 2014). The coarse-grained approach was used for simulation of highly complex systems containing many proteins, as exemplified by intracellular molecular crowding environments (Feig et al. 2017). The approach should be useful for MD simulation of whole IgG molecules and their complexes, given that appropriate pseudo-atom sets can be established that approximate amino acid and sugar residues.

MD simulation results, especially results obtained by coarse-grained methods, heavily depend on calculation protocol. In addition, longer MD simulations generally generate cumulative errors in numerical integration. Such error can be minimized with proper choice of parameters and algorithms, but it cannot be eliminated. Therefore, appropriate experimental validation of simulation results is necessary. In MD simulations of oligosaccharides, NMR paramagnetic effects were successfully used for experimental validation (Kato and Yamaguchi 2015; Kato et al. 2018a).

Dynamic interplay of the protein and carbohydrate parts revealed by integrative experimental and computational approaches

The pair of N-glycans linked to the Fc portion of IgG critically affects effector functions through modulation of interactions with $Fc\gamma Rs$ and complement component C1q (Yamaguchi et al. 2007). These N-glycans are packed between the two C_H2 domains and interact with domain inner surfaces. X-ray crystallographic studies indicate that core and $Man\alpha1$–6 branches of glycans interact with inner surfaces of C_H2 domains, leaving $Man\alpha1$–3 branches projecting into the central space of Fc. Nonreducing terminal Gal residues at $Man\alpha1$–6 branches make extensive contact with several amino acid residues. Gal residues on $Man\alpha1$–3 branches are often invisible (or absent) in the crystal structures. NMR studies also revealed distinct mobilities of the two terminal Gal residues in solution (Yamaguchi et al. 1998; Barb and Prestegard 2011). Crystallographic data indicate that the intramolecular interaction network of N-glycans is rearranged on binding to $Fc\gamma Rs$. Stable-isotope-assisted NMR revealed that truncation of outer carbohydrate moieties indirectly affected functional conformation of the $Fc\gamma R$-binding lower hinge segments (Yamaguchi et al. 2006; Kato et al. 2010).

Recently, dynamic views of human IgG1-Fc were published after long-timescale all-atom MD simulations starting from crystal structures (Frank et al. 2014; Lee and Im 2017;

Yanaka et al. 2019a). An extensive MD simulation for 3 μs in total, which was experimentally validated by SAXS (Yanaka et al. 2019a), indicated that dynamic conformational ensembles of Fc encompass a range of crystallographic snapshots previously reported for both free and complex forms (Fig. 2a, b, c). Notably, major Fc conformers in solution derived from the MD simulation exhibited almost symmetric and stouter quaternary structures, in contrast to crystal structures. In the MD-derived conformational ensemble of Fc, the N-glycan transiently makes contacts with amino acid residues located on the C_H2 surface and with the other N-glycans, which are not observed in crystal structures (Fig. 2d). MD simulations also indicated that removal of either the N-glycans or the hinge segments from Fc resulted in expansion of the conformational space, with increases in populations of asymmetrically distorted quaternary conformations, which, in addition to the local conformational perturbations in the hinge region, may negatively affect the affinity of IgG-Fc for $Fc\gamma Rs$ due to conformational entropy loss. Thus, experimentally validated MD simulations suggest that the N-glycans restrict the motional freedom of C_H2 and endow quaternary structure plasticity through multiple intramolecular interaction networks.

The core fucosylation of N-glycans of human IgG1-Fc is of biopharmaceutical interest because defucosylation increases its affinity for $Fc\gamma RIIIa$. The increase in affinity results in a dramatic enhancement of antibody-dependent cellular cytotoxicity activity (Isoda et al. 2015). Crystallographic and mutational studies highlight the importance of the Tyr296 side chain of IgG1-Fc. This residue is directly involved in interactions with $Fc\gamma RIIIa$ and other $Fc\gamma Rs$ (Mizushima et al. 2011; Radaev et al. 2001; Kiyoshi et al. 2015). Long-timescale MD simulations showed that the largest populations of Tyr296 conformations are significantly different between fucosylated and defucosylated forms, and both considerably deviate from semi-outward conformations observed in crystal structures used as initial structures in the simulations (Yanaka et al. 2019a). The Tyr296 side chain mainly exhibits an inward conformation in contact with the core fucose (Fig. 3a). This contact was experimentally confirmed by observing NOE connectivity. In contrast, in the defucosylated form of IgG1-Fc, Tyr296 primarily exhibits an outward conformation that is favorable for $Fc\gamma RIIIa$ binding (Fig. 3b).

Besides IgG-Fc, extracellular regions of $Fc\gamma Rs$ have several N-glycosylation sites (Yagi et al. 2018a). $Fc\gamma RIIIa$ possesses five such sites: Asn38, Asn45, Asn74, Asn162, and Asn169. Among the N-glycans at these sites, the Asn45 and Asn162 glycans have negative and positive effects on IgG-binding affinity for $Fc\gamma RIIIa$ (Shibata-Koyama et al. 2009; Cambay et al. 2020). Crystal structures of the complex formed between nonfucosylated IgG1-Fc and the extracellular region of $Fc\gamma RIIIa$ with these two N-glycans indicate that interactions for the two glycoproteins are mediated not only through
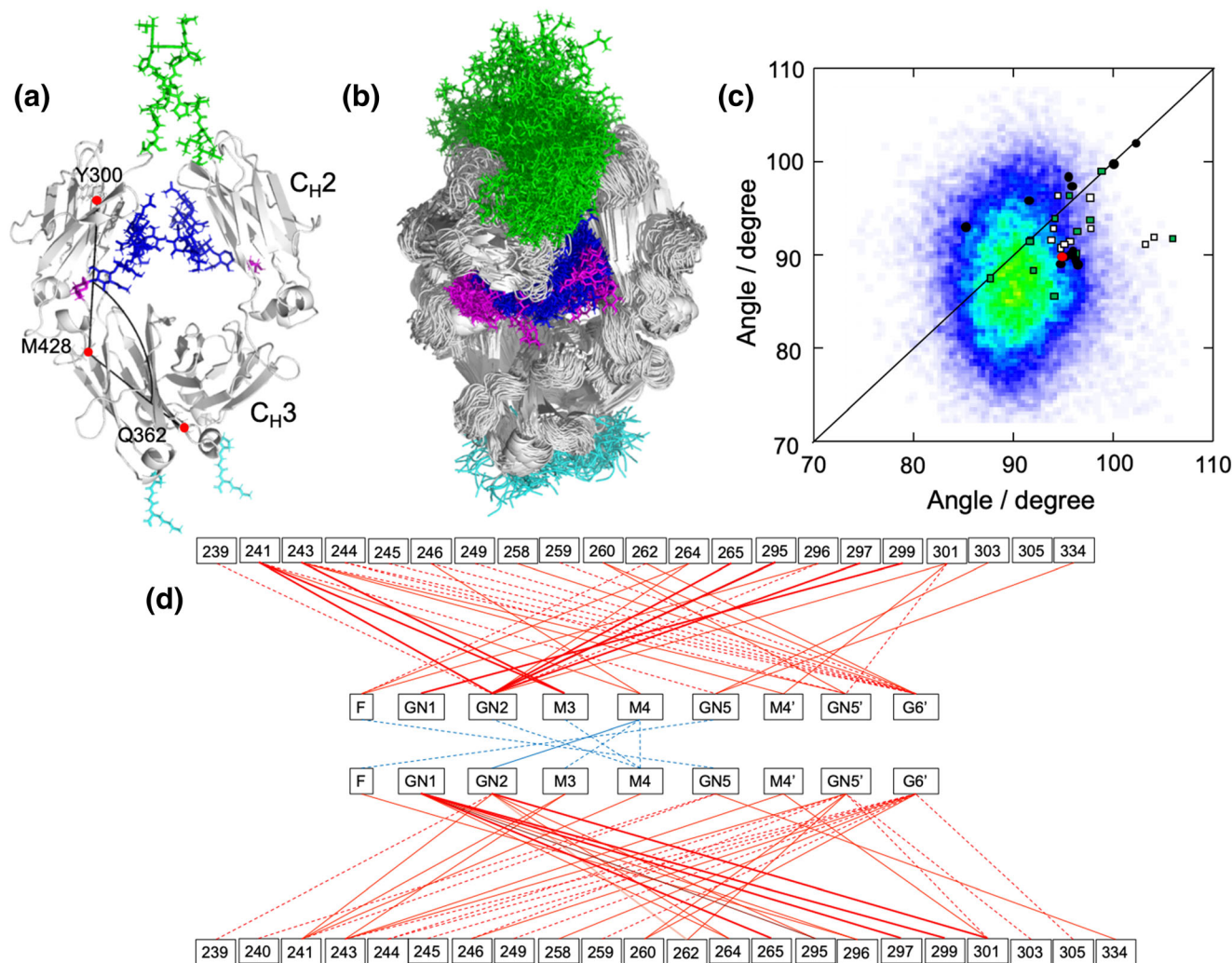
Fig. 2 Dynamic view of IgG-Fc provided by experimentally validated computational approach. (**a**) The starting structure of SAXS-validated MD simulation based on the crystal structure of fucosyl IgG1-Fc (3AVE) supplemented with the hinge (green) and C-terminal (cyan) segments along with the terminal galactose residues (magenta) of the Manα1–6 branches. N-glycans are depicted in blue except for the terminal galactose. The intra-chain domain-orientation angle between C_H2 and C_H3 defined by Cα atoms of Y300, M428, and Q362 are shown in chain A. (**b**) The superposition of 256 structures extracted every 100 ns from the MD trajectory. The structures were visualized by PyMOL (https://www.pymol.org). (**c**) Distribution of intra-chain domain-orientation angles between C_H2 and C_H3 for ensemble models of IgG1-Fc and various crystal structures of IgG1-Fc. Angles between C_H2 and C_H3 domains of chain A and chain B are plotted on the x and y axes, respectively. Data depict results from ensemble models derived from MD simulations along with angles observed in crystal structures [circles for uncomplexed Fc (red, the starting structures used for the corresponding MD simulations; black, Fc with native N-glycans), rectangles for complexed Fc structures (white, complex with sFcγRs; green, complex with other ligands)]. (**d**) Pairs of contact residues found within 4 Å in the ensemble model derived from the MD simulation are connected by different types of line segments (red for carbohydrate-protein contact and cyan for carbohydrate-carbohydrate contact) according to incidence as follows: more than 24,000 pairs (thick solid line), 24,000 to 16,000 pairs (thin solid line), and 16,000 to 8000 pairs (dashed line). Adapted from reference (Yanaka et al. 2019a) with modification

protein-protein interactions but also carbohydrate-carbohydrate interactions between the FcγRIIIa N162 glycan and one of the Fc N297 glycans (Mizushima et al. 2011; Ferrara et al. 2011) (Fig. 3c). In contrast, the N162 glycan of FcγRIIIa was largely invisible in crystal structures of its complex with fucosylated IgG1-Fc. This glycan is apparently not stabilized and remains mobile in the complex (Fig. 3d). REMD simulations based on these crystal structures allowed exploration of conformational dynamics of the four N-glycans were explored by extrapolating the missing information (Sakae et al. 2017). The Asn162 glycan is stabilized in solution mainly through interaction with the nonfucosylated form of Fc glycan. The glycan is, however, sterically hindered by the core fucose of Fc, rendering the Asn162 glycan more distal from Fc and more mobile.

Core fucosylation disrupts optimum intermolecular carbohydrate-carbohydrate interaction and restricts motional freedom of the Tyr296 side chain. This understanding explains how defucosylation increases the affinity of IgG1-Fc for FcγRIIIa and leads to improved efficacy of therapeutic
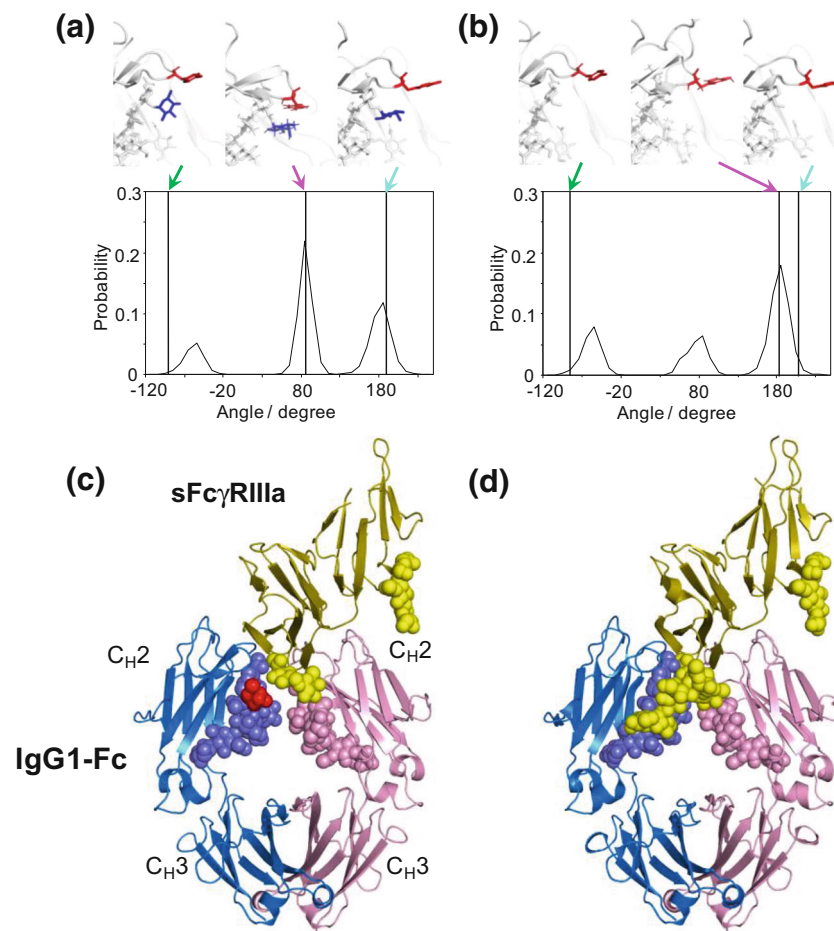
Fig. 3 Effects of core fucosylation of the Fc N-glycans on conformational dynamics of Tyr296 of IgG1-Fc and the N162 glycan of FcγR. Distributions of χ1 dihedral angles of Tyr296 in the ensemble models derived from MD simulations are plotted for (**a**) fucosylated IgG1-Fc and (**b**) nonfucosylated IgG1-Fc. The typical conformational snapshots of derived from the major conformational states (magenta arrows) in the simulation trajectory are shown along with the crystal structures used for building the starting models (green arrows; A, 3AVE; B, 2DTS) and those of sFcγRIIIa-bound Fc (cyan arrows; A, 5XJE; B, 3AY4). Crystal structures of (**c**) fucosylated and (**d**) nonfucosylated Fc complexed with

bis-glycosylated soluble form of FcγRIIIa (sFcγRIIIa). Chains A and B of Fc are blue and pink, respectively; sFcγRIIIa is yellow. Carbohydrates are represented as spheres. Fuc is colored in red. In the complex of fucosylated Fc, the N162 glycans of sFcγRIIIa gave electron densities only for the reducing-terminal residues, in marked contrast to the observation of the same sFcγRIIIa in complex with nonfucosylated Fc, in which the N162 glycan is extensively involved in interactions with the Fc glycan. Adapted from reference (Yanaka et al. 2019a) and (Sakae et al. 2017) with the modification

antibodies. These studies highlight the importance of integration of experimental and computational approaches for elucidating molecular mechanisms underlying glycosylation-dependent effector function promoted by interactions between IgG1 and FcγRIIIa.

Future perspective

A variety of experimental and computational tools are now available for characterizing conformational dynamics of antibodies. To date, antibody functions have been assigned to individual parts in the molecule. Also, the atomic-level analyses based on NMR spectroscopy and MD simulation have been used to focus on specific parts of antibodies. Antibodies

exert their multiple functions in a highly coordinated manner, as best exemplified by the interlocking of promotion of effector functions with antigen recognition (Yang et al. 2017). Recent HS-AFM data illustrate that IgG molecules bound to antigenic membranes are self-assembled into a hexameric ring and thereby recruit C1q (Yanaka et al. 2019b; Yogo et al. 2019). Moreover, Fab portions of IgG contribute to its interaction with FcγRIII (Yogo et al. 2019). Thus, a more integrated view of conformational dynamics of the entire antibody molecule is necessary for understanding putative allosteric mechanisms underlying antibody functions. Experimental and computational identification of correlations of local conformational dynamics will lead to elucidation of an intramolecular interaction network and interrelated allosteric pathways in the antibody molecule. Further, antibodies function

in heterogeneous bloodstreams, characterized by molecular crowding and promiscuous intermolecular interactions (Yanaka et al. 2017b). Improvements in individual techniques and their integration will promote understanding of dynamic behaviors of antibodies under varying physiological and pathological conditions. This understanding will provide opportunities for engineering antibodies through control of allosteric networks of IgG molecules.

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