# 3D molecular visualization of a human antibody by MAXS measurement reveals significant differences between the solution and crystalline states

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**Abstract**

X-ray solution scattering experiments have been utilized to analyze structures and conformational changes of biological macromolecules. Those experiments employ X-ray scattering data in a small-angle region, a technique called Small Angle X-ray Scattering (SAXS). SAXS experiments often focus on the macroscopic shapes and sizes of molecules. However, in principle, the scattering data in the higher scattering angle region contains more detailed structural information. For example, scattering data corresponding to q values ($=4\pi \sin\theta/\lambda$) between 0.30 and 0.65 $Å^{-1}$ reflects distances among domains, secondary structure modules, and/or adjunct chemical groups in Antibody–Drug Conjugates (ADCs). These Middle Angle X-ray Scattering (MAXS) experiments should give us a novel picture of complex molecular behaviors, as well as conformational changes in flexible biomolecules. In this article, we will discuss solution structure analysis of human Immunoglobulin G (IgG) by MAXS that revealed significant differences between the solution and crystalline states, as well as a novel observation of its flexibility.

## 1. Introduction

There has been an increase in the use of biopharmaceuticals, especially antibody drugs. While they have the advantage of higher specificity, and therefore weaker side effects, they have the disadvantage of expensive development and production costs. Moreover, maintaining and controlling their quality is problematic, since they consist of extensive protein moieties with complex molecular nature. The complexities come from both chemical and physical characteristics and, additionally, from their heterogeneities. The chemical heterogeneities come from spontaneous degradation; charge variants are one cause, and biochemical heterogeneity is exemplified by sugar chain variation. Such chemical characteristics have been well identified and controlled; however, the rapid expansion of novel modalities is making it more challenging to develop analytical methods for novel antibody-based drugs.

In contrast, most analytical methods for physical characterization tolerate the variations in those novel modalities. However, many of these are time-consuming and sample-demanding processes. Moreover, the results are difficult to manage and limited in use because essential information for physical properties—that is, the three-dimensional structures—are unavailable. X-ray crystallography and single particle cryo-EM observation are the established methods to explore the tertiary structure of biomolecules. However, those results correspond to the structure in constrained conditions and are probably affected by the crystallization or freezing process, as well as being limited to the specific subgroup of molecules intentionally selected from the original ensemble. Those limitations come from the flexible nature of biomolecules. Antibodies are particularly highly flexible, mainly at the Fab-Fc hinge and the essential sugar chains. This flexibility makes crystallization of whole antibody molecules unrealistic and/or impedes getting structural insight from frozen snapshots.

Having a method to visualize flexible biomolecules that avoids prerequisites for X-ray crystallography and structural constraints caused by the forces will undoubtedly be of great help in developing novel modalities, their CMC (Chemistry, Manufacturing and Control) studies, and quality control. X-ray solution scattering experiments have been utilized to analyze structures and conformational changes. In those experiments, scattering data within a small-angle region are widely used, a technique called SAXS (Small Angle X-ray Scattering). SAXS experiments often focus on the macroscopic shapes and sizes of the molecules. However, in principle, the scattering data in the higher q region contains detailed structural information that could be extracted; for example, q values between 0.30 and 0.65 $Å^{-1}$ reflect distances between domains, secondary structure components, and/or adjunct chemical groups in ADCs. These experiments should give us a novel picture of complex molecular behaviors, as well as conformational changes of flexible biomolecules.

In the above background, we are essentially working on the molecular visualization of medicinal macromolecules utilizing extensive SAXS data reaching into the sub-WAXS region. We call this approach MAXS, an abbreviation of Middle Angle X-ray

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Scattering. As shown in this article, MAXS experiments give us novel and valuable information that could lead to designing molecules and establishing production processing of antibody drugs. Here, as the first step of our work, we will discuss the mechanism of the flexible nature of IgG on its function. We performed solution structure analysis of IgG with and without sugar chains to visualize the difference in conformational characteristics, especially in the Fab regions. Deglycosylated IgG is known to have a significantly decreased binding capability to the Fc receptor, and the solution state that MAXS experiments can reveal may help to elucidate the reason for this.

## 2. Experiment

### 2.1. Human serum IgG

We investigated the capability of MAXS to measure and analyze flexible natural molecules, specifically human serum IgG. We also evaluated the effects of the sugar chain moiety in the Fc region on the structural nature of human IgG.

### 2.2. MAXS measurements and analysis

The MAXS experiments were performed at room temperature on a BioSAXS-2000 system equipped with a Rigaku FR-X Cu$K\alpha$ rotating anode X-ray generator and a photon counting X-ray detector at a camera distance of approximately 500 mm (Fig. 1). The scattering vector range was set from $q_{min} = 0.0080\,Å^{-1}$ to $q_{max} = 0.6600\,Å^{-1}$.


**Fig. 1.** The MAXS measurement system.

The final scattering curve was radially averaged with the SAXSLab (Rigaku) program. Subsequent data were analyzed by the ATSAS package[1]. The ab initio electron density maps were calculated from the MAXS scattering intensities using the program DENSS[2]. The electron density maps were depicted with PyMOL[3].

## 3. Results and Discussion

### 3.1. Flexible nature of human serum IgG

Antibodies are Y-shaped molecules, but their conformational movements have not been well studied. Antibodies are highly flexible; thus, it is challenging to represent an antibody's entire structure with only a single static model. Although several full-length antibody crystal structures have been determined, they are snapshots reflecting the restricted environment in the crystal and thus lose some essential characteristics in a solution that we need to know.

Therefore, we performed a 3D particle electron density analysis of human serum IgG using DENSS employing MAXS datasets. Figure 2a shows that the solution structure drastically differs from the crystal structure of IgG (PDB ID = 1hzh). Compared to the crystal structure, IgG in solution was extensively spread out in both Fabs regions, indicating that the crystal structure is much more compact in that environment. To evaluate the degree of restriction caused by crystal packing, crystal structures from three different antibodies (PDB ID = 1hzh: red, 1igt: blue, 1igy: green) were superposed. As shown in Fig. 2b, they are nearly identical, showing only minor fluctuations in the Fab and Fc regions, thus showing commonly compact states in the crystal. On the other hand, IgG has greater structural freedom in solution, and there are drastic differences in structural characteristics when IgG actually functions, showing the essential importance of solution structure analysis for highly flexible molecules such as antibodies. Next, we performed a detailed comparison of the solution and crystal structures of

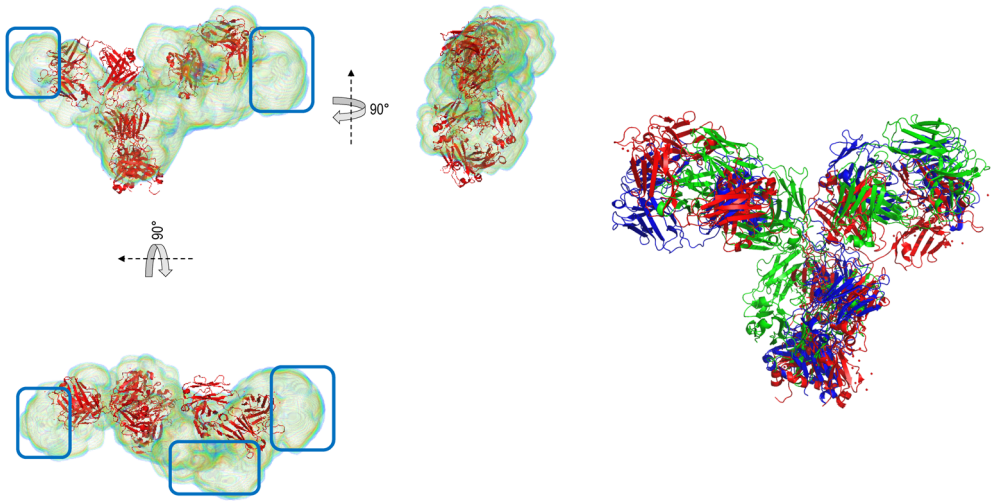


**Fig. 2.** Superposed images. (a) 3D particle density and crystal structure: PDB ID = 1hzh. (b) Three crystal structures: PDB ID = 1hzh: red, 1igt: blue, 1igy: green.

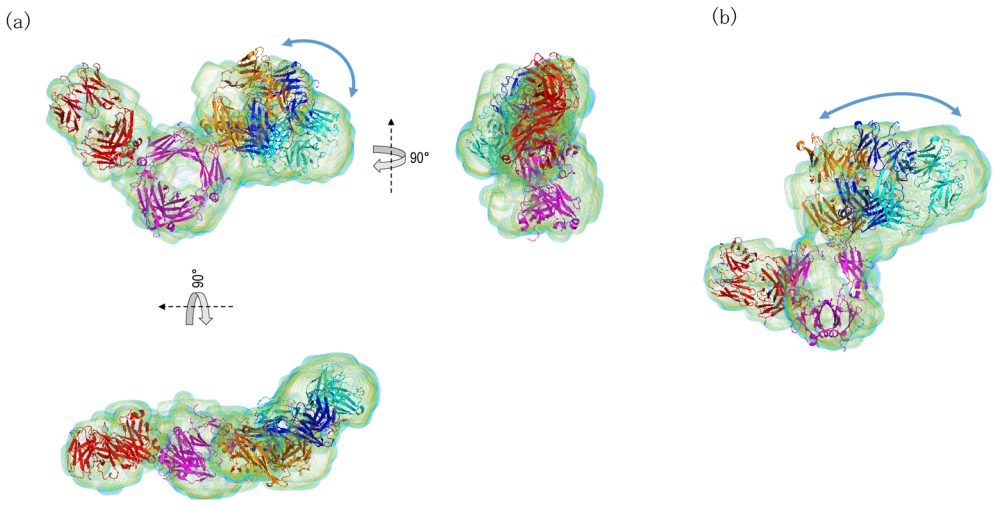


**Fig. 3.** (a) Fitting the domain structures observed in the crystals into the electron density map obtained by MAXS experiments of IgG. (b) Enlargement of Fab region, where the most significant discrepancy was observed.

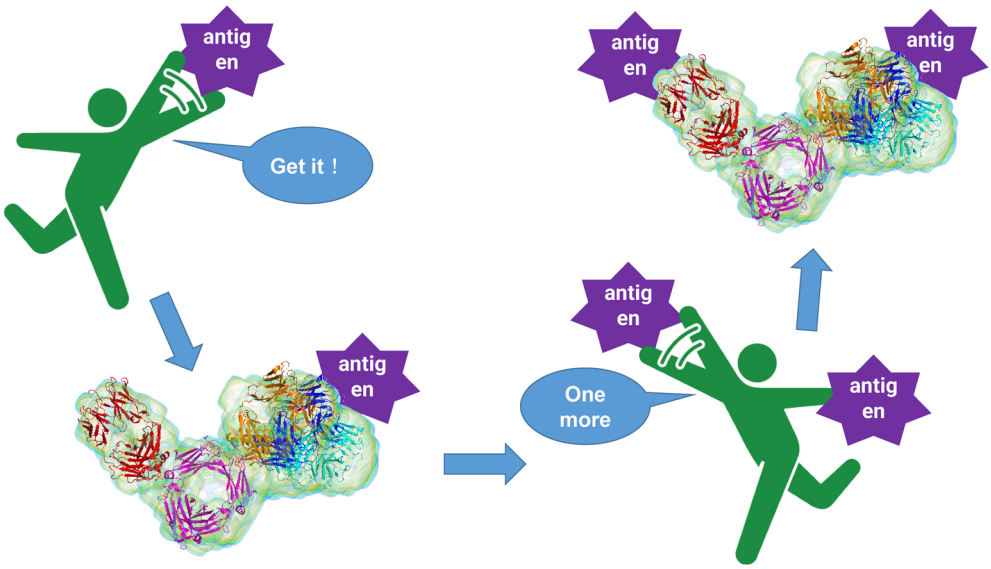


**Fig. 4.** The antigen-searching mechanism proposed by MAXS observation.

IgG by fitting the crystal structures of the Fab and Fc domains into the density map obtained by MAXS analysis using Coot[4] (Fig. 3a). Figure 3a shows excellent fit at one Fab arm, indicating that both crystal and solution states are very similar in this region. However, the electron density map at the other Fab arm showed a significant discrepancy. This suggests that one of the Fab arms has different characteristics between the crystal and solution state (Fig. 3b). These findings surprised us, because the two Fab arms in an antibody molecule are genetically identical and considered to behave equally. The spreading electron density at one Fab arm indicates high flexibility and uneven roles of the two Fab arms in an antibody molecule. We speculate that the antibody waves one Fab arm to efficiently focus on searching for antigens (Fig. 4). It might also be speculated that the sequential mechanism is like this: first, an IgG molecule travels around swinging one hand and captures the target antigen. Then, while holding the antigen by one hand, the IgG continues to move around to capture another antigen with the other hand.

### 3.2. The effects of the sugar chains

Sugar chains of antibodies play an important role in biological phenomena such as protein folding, transport, degradation, interaction, quality control, regulation of stability, and their functions. It is known that an IgG molecule lacking sugar chains has a significantly reduced ability to bind to the Fc receptor. This reduction is considered due to conformational changes caused by the loss of sugar chains. Borrok et al. compared the crystal structures of the Fc domain with and without sugar chains to determine what conformational differences are present[5] (Fig. 5, PDB ID=3ave: gray, 3s7g: red, 3dnk: blue, 3hkf: green). The distance between the C$\alpha$s of the $C_H2$ domain showed a closed

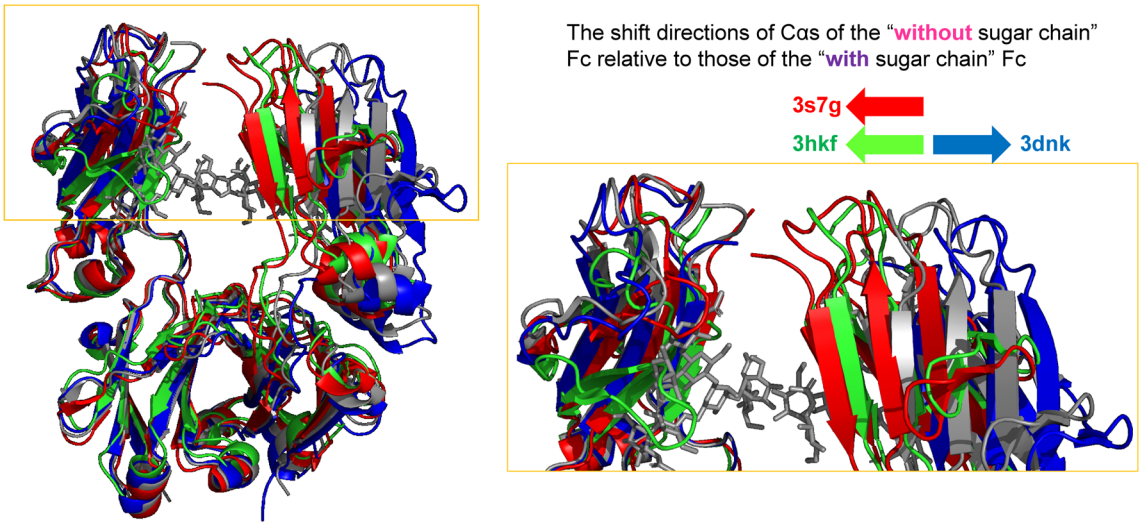


**Fig. 5.** Superposed Fc crystal structures of those molecules with and without sugar chains.

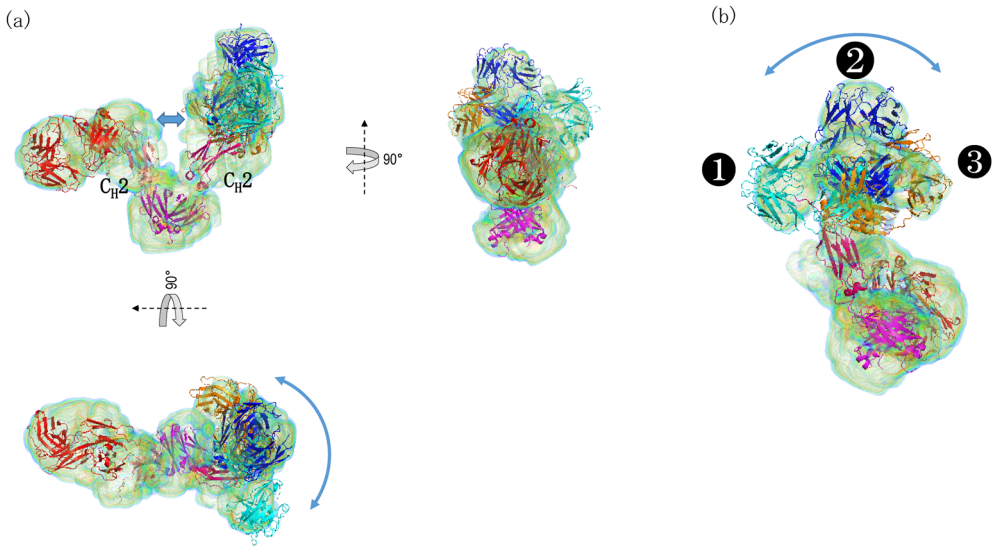


**Fig. 6.** (a) Electron density map of IgG without sugar chains and domain crystal structures superposed on the map. (b) Focused on Fab showing the mode of flexible movements.

conformation in the Fc without sugar chains, 3s7g and 3hkf, compared to the Fc with sugar chains, 3ave. However, the other deglycosylated Fc domain, 3dnk, showed an open conformation, indicating different results at 3s7g and 3hkf. This result indicates that the crystal structure shows visibly different structures from those obtained in the solution state. Moreover, loss of sugar chains in the Fc might increase the conformational freedom of the Fc domain susceptible to packing forces in the crystals. These results are not contradictory but converge into the observation as follows: the deglycosylated antibody molecule shows a more open structure than the original molecule and exhibits higher flexibility in solution, where no particular uneven forces exist to skew the molecule.

We have evaluated the effect of deglycosylation on the conformation of human IgG by employing MAXS measurements and 3D molecular visualization (Fig. 6a). The results revealed that the Fc of IgG without sugar chains showed an open conformation. Interestingly, the differences are more significant and prominent than the results observed in the $C_H2$ domains among all Fc crystal structures listed. These results might tell us the limitations of the crystal structure, the "frozen" snapshot in the crystal, on evaluations related to conformational changes or flexibilities in a molecule that has structural freedom in the solution state.

Regarding the Fab domain, uneven Fab movements were observed even in the deglycosylated molecule, similar to the intact antibody. Intriguingly, the observed unevenness in the Fab waving movements was more prominent in the deglycosylated molecule. This increased IgG mobility by deglycosylation is thought to be attributed to an increased degree of freedom of the $C_H2$ domain. The mode of movement was classified into the following three types of mobility, (1)–(2), (2)–(3),

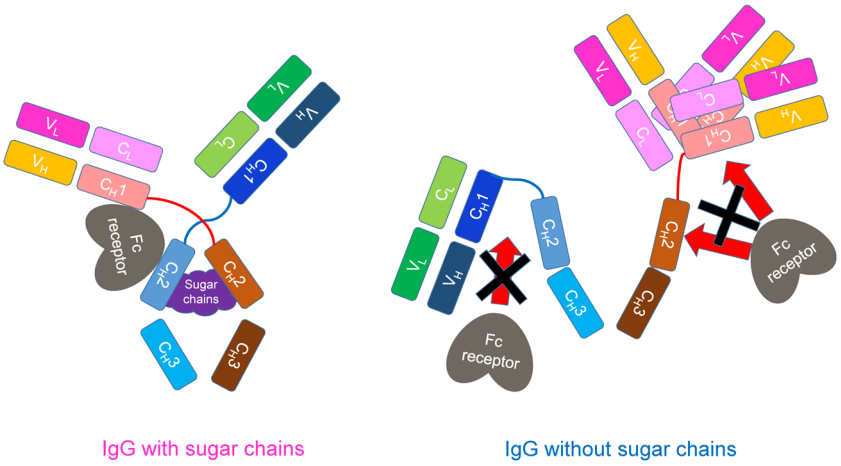


**Fig. 7.** Schematic drawing of the binding of IgG to the Fc receptor.

and (1)–(3), as shown in Fig. 6b.

Therefore, we considered that the cause of the decreased binding of the antibody to the Fc receptor is attributable to changes in conformational characteristics of IgG without sugar chains in the solution. First, we focused on the interaction region between IgG and the Fc receptor. Yogo et al. found that the Fc receptor interacts not only with the $C_H2$ domain of the Fc but also with the $C_H1$ domain of the Fab[6]. Accordingly, it is speculated that the relative positions, such as the distance and angle between the Fc and Fab, are important for binding IgG to the Fc receptor. Solution structure analysis of IgG with and without sugar chains shows that the distance and angle between the $C_H2$ and $C_H1$ domains are significantly changed. As shown in Figure 7, freedom of the $C_H2$ domain and Fab is increased, resulting in even greater flexibility of the Fab in IgG without sugar chains. As a result, the distance between the $C_H2$ and $C_H1$ domains is held closer together or further apart compared to IgG with sugar chains, resulting in a state where IgG without sugar chains is no longer suitable for binding to the Fc receptor.

On the other hand, on the opposite side, the $C_H2$ and $C_H1$ domains are in close proximity, suggesting that there may be insufficient space for the Fc receptor to interact. These findings suggest that both Fab sides of IgG without sugar chains caused a significant loss of ability to bind to the Fc receptor.

The cause of the decreased binding abilities of IgG without sugar chains to the Fc receptor can only be clarified after obtaining information on the heterogeneity of the distance, angle between the $C_H2$ and $C_H1$ domains, and the increased mobility obtained by 3D molecular visualization.

## 4. Summary

X-ray crystallography allows scientists to observe the detailed structure of molecules. However, to address the requirements of crystallization, highly flexible molecules are often modified or chopped into small domains to obtain a crystal. To evaluate structural characteristics correctly, it is necessary to employ the whole, intact molecule.

Based on the structures in the solution state, we found novel molecular features in antibodies, which are well-studied and utilized molecules. 3D molecular visualization with MAXS measurements provides the possibility to give information in solution, shedding light on highly flexible molecules such as antibodies. It may also contribute to functional improvements, quality evaluation, and stability assessment of biopharmaceuticals, including antibody drugs.

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