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Antibodies under pressure: A Small-Angle X-ray Scattering study of Immunoglobulin G under high hydrostatic pressure

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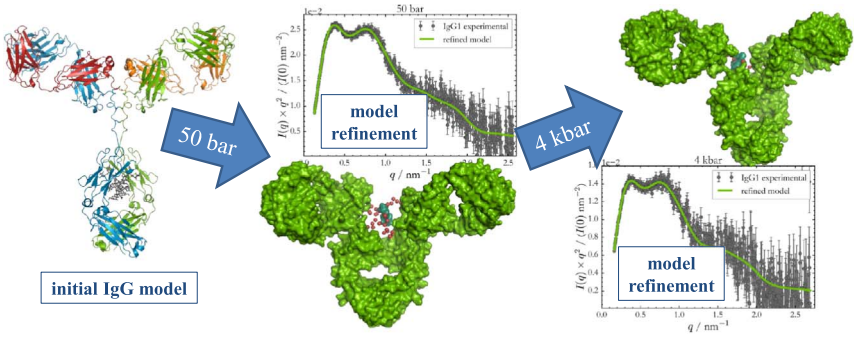
HIGHLIGHTS

- IgG solution structure differs considerably from available crystal structures.
- It withstands pressures of up to 5 kbar for at least several seconds.
- A symmetrical T-shape is maintained at up to 4 kbar.
- Experimental data can be reproduced computationally by a single IgG conformation.

GRAPHICAL ABSTRACT



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ABSTRACT

In the present work two subclasses of the human antibody Immunoglobulin G (IgG) have been investigated by Small-Angle X-ray Scattering under high hydrostatic pressures up to 5kbar. It is shown that IgG adopts a symmetric T-shape in solution which differs significantly from available crystal structures. Moreover, high-pressure experiments verify the high stability of the IgG molecule. It is not unfolded by hydrostatic pressures of up to 5kbar but a slight increase of the radius of gyration was observed at elevated pressures.

1. Introduction

The present work deals with the investigation of human Immunoglobulin G (IgG) antibodies in solution. Antibodies, also known as immunoglobulins, are a special class of proteins that play an important role in the immune system. IgG is the most abundant representative of this protein class. Via the commonly known lock-key-principle they bind to foreign particles that represent a potential harm to the organism and hence mark them for final clearance by other parts of the immune system. The high specificity and binding affinity of antibodies is exploited in several applications in the life sciences. Beside their extensive utilization in biological analytics (immuno assays), antibodies are more and more engineered by pharma industry as effective medical treatments, e.g. in cancer therapy [1]. At the current development rate, the therapeutic antibody market is expected to cross the 100 billion USD threshold by 2018 [2]. This gives rise to a lot of antibody-related research [3], much of it focusing on structural characteristics since those are particularly important for the correct function of antibodies [4].

The standard method in structural biology is macromolecular

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crystallography as it provides protein models at atomic resolution. But this method entirely relies on the production of high-quality protein crystals which is problematic for two reasons: firstly, crystallization of proteins is a highly non-trivial process [5]. And secondly, the protein is forced into a crystal lattice so that the crystal structure might deviate from the native solution structure. This concern is further enhanced by the fact that often additives are employed to facilitate crystallization while their effect on the protein structure is unknown [6]. These two objections particularly apply to flexible proteins like IgG. Due to IgG's inherent flexibility, it is rather unlikely that it adopts the same conformation in both crystal and solution. A technique more suited for the investigation of IgG is thus Small-Angle X-ray Scattering (SAXS) that allows to obtain unique structural information on large macromolecules in solution [7]. Hence, with the aid of advanced SAXS instrumentation and analysis software in recent years [8], a lot of SAXS studies on IgG were published lately, e.g. [9,10].

Whereas studies like the ones cited above explored IgG under a variety of experimental conditions like temperature, pH and protein concentration, the effect of high hydrostatic pressure on the protein structure remains a rather blank area on the map of antibody research. This topic is particularly interesting since there are a lot of applications of high pressure in the life sciences. For instance, when inclusion bodies form during recombinant protein production, the protein agglomerates can be dissolved via pressure application [11]. Another example is high-pressure processing as an alternative to traditional heat treatment to extend the shelf life of foodstuff [12]. In both examples IgG's pressure resistance is crucial. In the former because IgG-related pharmaceuticals are typically produced recombinantly and in the latter because this technique is already in use for milk products. Milk is naturally high in antibody content that is desirably preserved during processing.

In this study we show that high hydrostatic pressure has only a minor effect on the protein, yielding a small increase of the radius of gyration, while the overall native T-shaped structure in solution, which differs from crystallographic data, is conserved.

IgG is usually depicted as a Y-shaped molecule as shown in Fig. 1. It is a bilaterally symmetric, 150 kDa hetero-tetramer consisting of two identical light chains (25 kDa) and two identical heavy chains (50 kDa) that are linked by disulfide bridges. The light chains can be divided into a variable and a constant region whereas the heavy chains have one variable region and three constant regions. The variable regions of each a light and a heavy chain form the domains at the Y-tips where the antigen binding sites are located. The constant regions form in total four further domains. Digesting IgG by Papain splits the protein into the three parts of its Y-shape: the stem is called Fragment crystallizable (Fc) and the two identical arms are the Fragments antigen-binding (Fab). These fragments are linked by the so-called hinge region, a flexible part of the heavy chains.

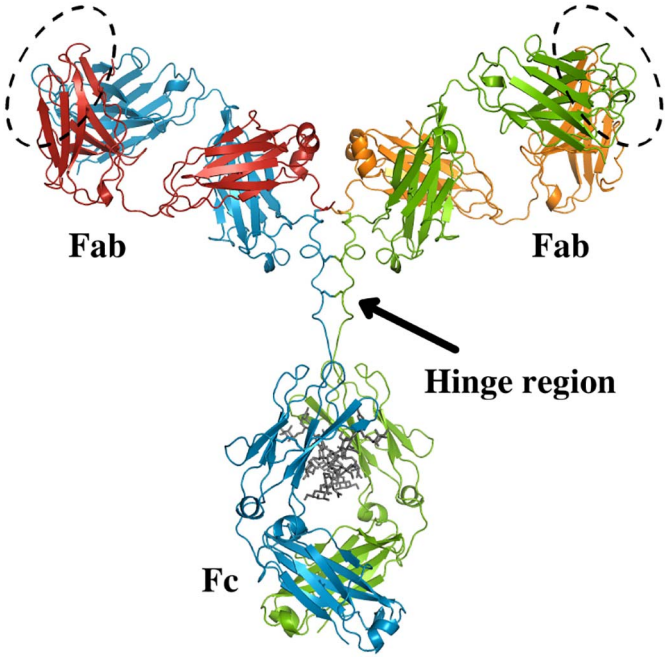

Fig. 1. Traditional Y-shaped conformation of IgG, based on the structure proposed by Padlan [13]. Antigen-binding sites marked by dashed ellipses.

There are four IgG subclasses named IgG1, 2, 3 and 4, which mainly differ in the number and location of their disulfide bridges and the length of their hinge regions. The differences originate from different heavy chain modifications. There are also two types of light chains, which slightly differ in shape [14] but seem to have no functional difference. In this study IgG1 and IgG2 antibodies were investigated. The main difference between the two heavy chain types is the length of their hinge region: where the IgG1 hinge contains 15 residues, the IgG2 hinge contains only 12 [15,16].

2. Experimental

Human Immunoglobulin IgG1 and IgG2, purified from pooled human blood, were purchased from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, and used without further purification. Both were shipped frozen in 20 mM Tris buffered saline, pH 8.0, kept frozen during transport and thawed in a refrigerator on the day of the experiment. Samples were concentrated to 1 to 8 mg/mL using Amicon Ultra centrifugal filters with a molecular weight cut-off of 30 kDa (Merck Millipore, Darmstadt, Germany). The respective flow-through was used for background measurements.

The experiments were performed at beamline ID02 at the ESRF (Grenoble, France). In SAXS experiments, the elastically scattered X-ray photons are detected in dependence of the wave vector transfer q, defined as $q = 4\pi\sin\theta/\lambda$ where 2θ is the scattering angle and λ the X-ray wavelength. Here, a photon energy of 17 keV was used. Scattering patterns were recorded with a 170 mm × 170 mm Rayonix MX-170HS CCD pixel detector at 2 m sample-detector distance, resulting in a wave vector transfer range of about $q = 0.05\ \mathrm{nm}^{-1}$ to $5\ \mathrm{nm}^{-1}$. Furthermore, water was used as reference for the absolute calibration of the scattering intensity. For in situ high-pressure experiments a custom-built SAXS sample cell [17] was employed.

To avoid radiation damage, a test-sample was exposed to the beam multiple times at 50 bar until radiation damage occurred, visible in a change of the scattering pattern. The maximum endurance was equally divided onto the number of frames needed for the planned pressure series, resulting in ≤1 s exposition time per frame. A typical pressure series started at 50 bar and was continued in 1 kbar steps up to 5 kbar. The samples were given several seconds for equilibration at each pressure step. Every series was followed by a measurement of the pure buffer solution at the same pressures for proper background subtraction. The measurements were repeated on freshly prepared samples in order to enhance the data statistics.

3. Data analysis and discussion

In the first step of data analysis the background was subtracted from the scattering data. The SAXS data of a typical pressure series is shown in Fig. 2. The overall scattering intensity decreases with increasing pressure, due to a loss in electron density contrast. The pressure-induced rise in water density results in the electron density of the aqueous phase approaching that of the proteins and therefore the contrast diminishes. Beside that, the main features of the scattering curve seem to be conserved.

Applying Guinier's law, the radius of gyration R_g was obtained from a linear fit to the data in Guinier representation up to the empirical validity limit $q_{\max} \times R_g \leq 1.3$. Here $q_{\max}$ is the maximum q-value used in the refinement process. The fit results of the pressure series, averaged over the multiple respective datasets of IgG1 and IgG2, are shown in