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Global structures of IgG isotypes expressing identical variable regions

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Abstract

Until relatively recently the immunoglobulin molecule was viewed as composed of two independent domains comprised of the variable (V) and constant (C) regions. However, recent work has established that the C region mediates allosteric changes in the V region that can influence specificity and affinity. To further explore cross-domain interrelationship in murine IgG structure we carried out solution small angle X-ray scattering (SAXS) measurements for four V region identical IgG isotypes. SAXS analysis revealed elongated Y-shaped structures in solution with significantly different, isotype-dependent domain orientations. To further explore local C region effects on the V region, the IgG₃ Fab crystal structure from the same family was determined to 2.45 Å resolution. The IgG₃ Fab crystal structure differs from a closely related previously solved IgG₁ Fab revealing significant structural differences, which may account for isotype-related specificity differences in V region identical Abs. Among the four murine isotypes, IgG₃ was the most different in solution with regards to overall structure as well as aggregate formation in solution suggesting that the greater apparent affinity of this isotype resulted from polyvalent complexes with enhanced avidity. Our results provide additional evidence that Ig V and C domains influence each other structurally and suggest that V region structure can have significant effects on overall Ig structure.

Keywords

Immunoglobulin; Isotype; SAXS; X-ray crystallography; Variable domain; Constant domain; Antigen

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1. Introduction

Immunoglobulin molecules are products of the adaptive humoral immune response that function as antimicrobial proteins. Igs are heterodimers in which each monomer contains two polypeptide chains, a heavy (H) and a light (L) chain. Each H and L chain has a C region that defines the class, or isotype of the antibody (Ab), as well as a V region that provides the interface that directly interacts with the Ag, also known as the paratope. The C region of an IgG molecule, which is the most prevalent form of Ig in both human and murine sera, is composed of the CH1–CH3 domains, which are located in both the Fab and Fc regions. The C region defines the isotype and confers effector properties such as interactions with Fc receptors, half-life, and complement actions (Ravetch and Kinet, 1991). In mice there are four IgG isotypes that have been found to be responsible for the identification and clearance of many peptide and polysaccharide antigens (Ags): IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ (Snapper and Mond, 1993). Interactions of IgG-Ag complexes with other cells of the immune system include Ab-dependent cytotoxicity (ADCC), phagocytosis, regulation of lymphocyte proliferation and Ab secretion. These effector functions are mediated through the interactions of Fc domains of IgG Ab or immune complexes with specific receptors on immune cells known as the Fc γ receptors (Fc γ Rs) (Ravetch and Kinet, 1991).

In recent years several laboratories have reported that the specificity and affinity of Abs can be influenced by the C regions such that these properties are not solely the purview of V region structure (Cooper et al., 1993; Janda et al., 2012; McLean et al., 2002; Pritsch et al., 2000; Torres and Casadevall, 2008; Torres et al., 2005, 2007b; Tudor et al., 2012; Xia et al., 2012). The mechanism for this effect presumably involves isotype dependent influences on V region secondary structure following antigen (Ag) binding (Janda and Casadevall, 2010). Furthermore, IgG molecules express different electronic emission spectra and proteolytic capacity (Janda et al., 2012). Much is known about the crystal structures of both Fab and Fc regions of Ig molecules, as separate domains. Each region is composed of two or three domains of anti-parallel β -sheets that form β -barrel domains (Davies et al., 1975). Although a few groups have been able to crystallize entire Ig molecules, there are very few complete structures from those crystals (Saphire et al., 2002). The first two were human myeloma IgG₁ molecules, Mcg (Edmundson et al., 1970) and Dob (Terry et al., 1968) whose sequences do not have hinges or intra-light chain disulfide bonds that occur in normal IgGs. Furthermore, due to the hinge deletion, Mcg and Dob are conformationally restricted and have little to no effector function, and their crystal structures were compact, T-shaped and symmetric (Saphire et al., 2002). Two other human IgG₁ structures, Kol (Huber et al., 1976) and Zie (Ely et al., 1978) with full-length hinges, failed to provide ordered electron density in their Fc domains (Saphire et al., 2002). Only three full-length IgG crystals thus far have been able to provide a complete picture involving all three IgG domains. The first is murine anti-canine lymphoma 231 IgG_{2a} (PDB: 1IGT) (Larson et al., 1991), the second is murine anti-phenobarbital 61.1.3 IgG₁ (PDB: 1IGY) (Harris et al., 1998) the third is human b12 IgG₁ anti-HIV-1 gp120 (Saphire et al., 2001, 2002) (PDB: 1RHH). There is significant variation in the orientation of the domains of these structures, suggesting that a considerable degree of flexibility exists within IgG molecules (Harris et al., 1998; Saphire et al., 2002). This flexibility, most of which occurs around the hinge region of the Ab molecule, makes IgGs difficult candidates for crystallographic studies. Hinge flexibility also raises the possibility that structures obtained by X-ray crystallography are snapshots of a single conformation (Sawaya and Kraut, 1997), such that in solution and *in vivo*, there may be considerable dynamic flexibility.

SAXS is a powerful technique that gives information about the size, shape, surface-to-volume ratio, distribution and oligomerization of molecules in solution (Bernado and

Svergun, 2012). Previously SAXS data involving IgG molecules has been collected on a number of human IgGs (Abe et al., 2010; Ashish et al., 2010; Gregory et al., 1987; Kilar et al., 1985; Lu et al., 2006; Rathore et al., 2011), as well as one murine IgG – a family of anti-dansyl isotypes using whole IgGs (Igarashi et al., 1990). Igarashi et al. collected SAXS data on a family of murine IgG switch variants: IgG₁, IgG_{2a}, IgG_{2b} and an IgG_{2a} with a C_{H1} deletion (referred to from now on as IgG_{2a(s)}). The radii of gyration (R_g) values that they found for these mAbs ranged from 50.7 to 53.9 Å, which are within the same range as those found for human IgGs, and the hinge angles were IgG_{2a(s)}, 15°; IgG₁, 180°; IgG_{2a}, 100° and IgG_{2b}, 120° (Igarashi et al., 1990). These hinge angles are not similar to those that have been identified by X-ray crystallography for the murine 231 IgG_{2a} (PDB: 1IGT) which is 172°, and for the murine 61.1.3 IgG₁, which is 115° (Saphire et al., 2002). These differences in hinge angles, obtained by different techniques, may be attributed to the flexible behavior of hinge regions. Flexible hinge regions facilitate an ensemble of conformations of Fab–Fc hinge orientations. X-ray crystallography may capture one or few of these conformations depending on how many molecules with different conformations are present in the asymmetric unit, in addition crystal contacts may also affect Fab–Fc orientations. Consequently, there is a need for studies to further delineate the structure of IgGs in solution, and on a set which has been at least in part characterized at the atomic level by crystallography.

Elucidating the mechanisms by which the C region can affect the specificity, affinity and avidity of IgGs is critical to better understand the Ig molecule, isotype switching and new possibilities in Ig engineering. To this end, we have first determined the global envelope structures of four murine IgG isotypes with solution SAXS to infer isotype-dependent global structural differences, and second, we have determined the IgG₃ Fab crystal structure to 2.45 Å resolution and compared it to a previously published IgG₁ Fab crystal structure from a related IgG₁ to elucidate structural differences imposed by C regions in atomic resolution. Furthermore, using all atom molecular dynamics simulations, we have modeled the 3E5 IgG₃ Fab domain bound to the peptide antigen P1. Hence, this paper includes two partially independent stories related to isotype structural variability – the comparison of four IgG isotype solution structures and the crystallographic comparison of two Fabs arising from the same V region genes. The results provide insight to the mechanism by which the C region affects the affinity, avidity and specificity of the V region.

2. Materials and methods

2.1. mAb preparation

The IgG switch variants of 3E5-IgG₃ have been described previously (Dam et al., 2008; Torres et al., 2007a). The murine mAbs were secreted by hybridoma cell culture supernatants and purified by protein A or G affinity chromatography (Pierce). mAbs were concentrated and buffer exchanged against 0.1 M Tris–HCl, 0.05 M NaCl pH 8.0. mAb concentration was determined by absorbance at 280 nm using an E value of 1.35. 3E5 IgG₁ and IgG₃ samples were monodisperse by Sephacryl-200 high pressure liquid chromatography, the other isotypes were not tested (data not shown).

2.2. X-ray scattering

X-ray scattering was obtained during a single beam session at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratories (BNL), New York on the X9A beamline. Details of the instrument configuration are published elsewhere (Allaire and Yang, 2011). All IgGs were 1–2 mg/ml, in samples with volumes of 15 µl each. Measurements were taken in flow cells to reduce radiation damage. Four frames of 30 s each were taken for each sample. Measurements were checked continuously for radiation damage

and then averaged. The reduction of the scattering data was done by correcting the data for buffer scatter and intrinsic anomalies specific to the experimental set up, *i.e.* detector inhomogeneity and dark noise using standard data reduction software, PyXS at NSLS X9A beamline.

2.3. Analysis of reduced scattering data

The analysis was done by utilizing GNOM (Svergun, 1999), which evaluates the pair-distance distribution function, $P(r)$, and calculates the radius of gyration, R_g , from one-dimensional scattering curves for mono-disperse systems. The scattering length unit $2\pi \sin \theta/\lambda$ was used with λ in Å. The low-resolution *ab initio* envelopes of Igs were calculated from their respective scattering profiles using DAMMIN (Svergun, 1992). DAMMIN uses simulated annealing for global minimization of the discrepancy (χ), between the experimental scattering curves and those that were calculated from dummy atom models. Different scattering angle, q (Å⁻¹), and distance, R (Å), ranges were used for individual Ig runs; IgG₁ ($q = 0.013$ – 0.080 , $R = 1$ – 175), IgG_{2a} ($q = 0.017$ – 0.113 , $R = 1$ – 175), IgG_{2b} ($q = 0.015$ – 0.078 , $R = 1$ – 175) and IgG₃ ($q = 0.013$ – 0.095 , $R = 1$ – 185). The q and R pair, which gave the best χ value in DAMMIN fast mode calculations, was used for the production runs, which were done in DAMMIN slow mode. In production runs, 10 individual models were generated for each Ig; P1 symmetry (no symmetry) was imposed in all calculations and a sphere was used as initial model. The calculations gave excellent χ values for IgG₁, IgG_{2a} and IgG_{2b} with χ^2 values of 1.06, 1.45 and 0.98 respectively. IgG₃ suffered from an inherent propensity of its Fc regions to aggregate (Greenspan and Cooper, 1993), which limited the soluble mono-disperse concentrations during data collection, rendering a mediocre quality fit and a χ^2 of 29.20. Ten DAMMIN models were then subjected to envelope averaging using DAMAVER (Volkov and Svergun, 2003), where models were compared pairwise resulting in a normalized spatial discrepancy (NSD) value for each model. The model with the lowest NSD value was considered to be the most accurate model and SUBCOMB (Kozin and Svergun, 2001) was used to fit all other models onto this reference model. Finally DAMFILT (Volkov and Svergun, 2003) was used to filter low occupancy and loosely connected atoms, which generated a compact/most probable model.

2.4. Fitting high-resolution crystal structures into low-resolution envelopes

There are only two full-length murine IgG structures available, which are IgG₁ (PDB: 1IGY) and IgG_{2a} (PDB: 1IGT). In addition, there are several crystal structures available for IgG_{1,2a,2b} fragments; either Fab domains or Fc domains. To fit the high-resolution structures of IgG₁ and IgG_{2a} into respective low-resolution maps we divided the full-length crystal structures from hinge regions and fitted the fragments individually to the low-resolution envelopes. For IgG_{2b} and IgG₃ the individual Fab (PDB: 1MAM for IgG_{2b}) (Rose et al., 1993) (PDB: 4HD1 for IgG₃) and Fc (PDB: 2RGS for IgG_{2b}) (Kolenko et al., 2009) (no Fc crystal structure is available for IgG₃), fragments were fit into the low-resolution maps. During the fitting Fab and Fc fragments are considered rigid bodies. The fittings were done using UCSF-CHIMERA with global search algorithm with random initial placement. The search algorithm was instructed to generate 30 distinct fit models. The highest scoring models were inspected manually and chosen to represent the final model. Due to the low-resolution nature of the SAXS-generated *ab-initio* envelopes, the fitted models may not be the accurate representations of the actual topologies.

2.5. Crystallization and structure determination of 3E5 IgG₃ Fab

The crystallization was done by sitting drop vapor diffusion at 21 °C by mixing 1 µl of the protein with 1 µl of reservoir solution (0.1 M sodium acetate trihydrate (pH 4.5) and 25% (w/v) polyethylene glycol 3350) and equilibrating over 45 µl of reservoir solution. Crystals

were transferred to reservoir solution supplemented with 20% glycerol prior to flash-cooling in liquid nitrogen. X-ray data were collected on an ADSC QUANTUM 315 CCD detector at NSLS beam line X29A and processed with HKL2000 (Minor et al., 2006). Diffraction data from the crystal were collected at a wavelength $\lambda = 0.9791 \text{ \AA}$ and were consistent with space group, $P2_12_12_1$ ($a = 67.070$, $b = 98.289$, $c = 142.363 \text{ \AA}$), with two heterodimers per asymmetric unit. Molecular replacement was performed with MOLREP (Lebedev et al., 2008) using the previously determined structures of 2H1 (PDB ID: 2H1P), and 9–40 Fab (PDB ID: 1T66) as an initial search model for the heavy and light chain, respectively. Subsequent model building and refinement was performed with Coot (Emsley and Cowtan, 2004) and REFMAC5 (Murshudov et al., 1997). The final model was refined to $2.45 \text{ \AA}$ with $R_{\text{work}} = 0.208$ and $R_{\text{free}} = 0.264$ (Table 1).

2.6. Protein Data Bank accession number

Coordinates of 3E5 IgG₃ Fab have been deposited in the Protein Data Bank with accession number 4HDI (<http://www.rcsb.org/pdb/home/home.do>).

2.7. All atoms molecular dynamics simulations of 2H1

The GXM binding IgG₁ mAb 2H1, which differs from IgG₃ mAb 3E5 by 12 amino acids in the V regions (eight amino acids in the V_H and four amino acids in the V_L chains) and thirty-one amino acids in their C regions. Its cognate peptide antigen PA1 shares sequence similarity (GLQYTPSWMLVG) with peptide P1 (SPNQHTPPWMLK), which is the cognate peptide of 3E5 IgG₃. The peptide-complexed Fab crystal structure (PDB: 2H1P) is found for 2H1 + PA1. Using this crystal structure and performing mutations *in silico* on both 2H1 and PA1, a model for the 3E5-IgG₃ + P1 complex has been generated. A 10 ns all atom MD simulation has been performed on this complex at constant temperature and pressure (300 K, 1 atm) with AMBER11 (Case et al., 2010). AMBER99SB (Hornak et al., 2006) force-field was used with the TIP3P (Jorgensen et al., 1983) explicit solvent model in a rectilinear box of dimensions $110 \text{ \AA}$, $77 \text{ \AA}$, and $85 \text{ \AA}$. Prior to the 10 ns run, a short minimization followed by a 20 ps heating (0–300 K), a 20 ps density equilibration and a 100 ps constant pressure/temperature (1 atm/300 K) equilibration steps were taken. The MD trajectory was processed and the minimum energy structure was chosen for further analysis, this structure is called as IgG₃* + P1 hereafter.

3. Results

3.1. Small angle X-ray scattering

SAXS data revealed important structural features of full-length 3E5 IgG isotypes in solution. These features included distance distribution profiles, radius of gyration (R_g) and globular morphology of molecules; *i.e.* average positions and orientations of individual domains with respect to other domains. The experimental scattering curves of all 3E5 IgG isotypes, Guinier analysis and respective distance distribution profiles (P_R) calculated by GNOM (Svergun, 1992) are shown in Fig. 1. Distance distribution profiles for all 3E5 mAbs are skewed Gaussian functions with multiple peaks; either one major peak and a less intense shoulder or two equally intense peaks as in IgG_{2b}. The skewed Gaussian profiles indicate elongated particles. The biphasic profile for IgG_{2b} may indicate rigidity such that the Fab and Fc domains of IgG_{2b} sample discreet orientations that are not averaged during the acquisition time. The radii of gyration, R_g , are calculated by two different methods; (1) integrating P_R with r^2 over all values of r for individual Igs and (2) using Guinier analysis. Both analyses revealed similar R_g values for all Igs except IgG₃, which most likely reflect the inherent aggregation propensity of this isotype. In addition the Guinier analysis assumes a globular shape whereas the Igs are elongated particles. The R_g values obtained from P_R integration are $55.44 \pm 0.22 \text{ \AA}$ for IgG₁, $54.47 \pm 0.34 \text{ \AA}$ for IgG_{2a}, $53.52 \pm 0.32 \text{ \AA}$ for IgG_{2b}

and 57.82 ± 0.14 Å for IgG₃. The R_g values are similar for all isotypes, but it is notable that IgG₃, which is known to have only one disulfide contact in the hinge in contrast to three or four for the other isotypes, has the largest R_g . In addition, the maximum molecular dimensions (D_{max}) found for IgG₁, IgG_{2a} and IgG_{2b} were 175 Å whereas D_{max} of IgG₃ was 185 Å. These values are in close agreement with those reported previously on other murine IgGs (Igarashi et al., 1990).

Y-shaped morphology is a characteristic of immunoglobulin molecules, and *ab initio* models calculated using DAMMIN (Svergun, 1999) identified this morphology for all Igs (Fig. 2) with differing Fab–Fab and Fab–Fc angles. The Fab–Fab hinge angles approximated from the low-resolution maps, generated from these models using CHIMERA (Pettersen et al., 2004) are $152 \pm 9^\circ$ for IgG₁, $146 \pm 8^\circ$ for IgG_{2a}, $143 \pm 9^\circ$ for IgG_{2b}, and $154 \pm 10^\circ$ for IgG₃. The Fc–Fab hinge angles for these isotypes are $146 \pm 7^\circ$, $180 \pm 5^\circ$, $180 \pm 5^\circ$ and $106 \pm 7^\circ$ respectively. The standard errors were calculated using models generated by DAMMIN for individual Igs. DAMMIN generated 10 models for each Ig run. Previously reported high-resolution crystal structures of Fab (PDB: 1MAM for IgG_{2b}) and Fc (PDB: 2RGS for IgG_{2b}) domains or full length Igs (PDB: 1IGY for IgG₁ and PDB: 1IGT for IgG_{2a}) broken into their respective Fab and Fc fragments were then docked into the low resolution maps (Harris et al., 1997, 1998; Kolenko et al., 2009; Rose et al., 1993). In general, the Y-shaped morphology obeys P1 symmetry with two similar sized domains, which correspond to the two Ag binding arms, symmetric about the plane passing through a slightly larger domain, the Fc or C region arm. The Fab–Fab angles for all isotypes are similar while the Fab–Fc angles showed differences among the isotypes. IgG_{2a} and IgG_{2b} are closest in C region sequence, and the Fab–Fc angles for these two isotypes are identical. The Fab–Fc angles are significantly different for IgG₁ and IgG₃. IgG isotypes express identical V regions thus these differences seen in global solution structures are attributed to the alterations of sequence of the C regions.

3.2. X-ray crystallography of IgG₃ Fab

Like other murine IgG Fab molecules, 3E5 IgG₃ Fab (PDB: 4HDI) is composed of four β-barrel domains made from the light chain and heavy chain. Each chain forms two discrete sections of β sheets, which, when combined, lie anti-parallel with respect to each other. The Ag-binding surface is composed of six loops made of the L chain hypervariable regions (L1–L3) and the H chain hypervariable regions (H1–H3) (Fig. 3C). The structural CDRs were defined as described by MacCallum et al. and include residues 24–35 (L1), 55–61 (L2), 94–102 (L3), 31–35 (H1), 50–55 (H2) and 99–109 (H3) (MacCallum et al., 1996). These CDRs correspond well to the commonly used Kabat numbering scheme (Johnson and Wu, 2000). The electrostatic potential of the Fab binding surface (Fig. 3), calculated according to Coulomb's law using Chimera, shows a few positive (^LLys55, ^HArg99) and negative (^HAsp56) patches. These charges surround the innermost pocket created by the six CDR loops. The binding pocket with a few charged residues is primarily composed of hydrophobic residues that create hydrophobic cavities essential to Ag binding. The most solvent-exposed residues among the CDRs were ^LSer32, ^LAsn33, ^LGly34 (L1), ^LVal99, ^LPro100, ^LTrp101 (L3), ^HAsn31 (H1) and ^HSer53, ^HAsn54, ^HGly55, ^HAsp56 (H2) (Fig. 3E).

3.3. Comparison of 3E5 IgG₃ Fab and 2H1 IgG₁ Fab

To assess differences in structural features between 3E5 IgG₃ Fab (PDB: 4HDI) and 2H1 IgG₁ Fab (PDB: 2H1P), another mAb against *Cryptococcus neoformans* capsular polysaccharide GXM that uses the same V gene elements but whose V region sequences differ only by twelve amino acids as result of somatic mutation (Young et al., 1997), the two crystal structures were overlaid. Comparison of these two structures revealed that when

either the V domains or the C domains were overlaid, structural alignment was almost identical, with the exception of the elbow area between the V and C regions (Fig. 4). Stanfield et al. defines the elbow angle in a different way in their study in which they compared and statistically analyzed more than 300 Fab structures (Stanfield et al., 2006). For the simplicity of the discussion, and since we are only comparing the two related Fab structures rather than analyzing the statistical distribution of all Fab elbow angles of published structures, we defined elbow angle as the angle between two axes (Fig. 4A) with the first axis through the C region and the second through the V region, the point where these two axes intersect correspond to the elbow. The other two points necessary to define angles were chosen as the same V-region residues in Ag binding sites of both IgGs. With this definition the elbow angles were 36.6° for the 3E5 Fab and -11.4° for the 2H1 Fab. Thus, the 3E5 IgG₃ molecule appears to be more 'bent' at its elbow than the 2H1 IgG₁ molecule. This difference can be explained by several different mechanisms; first, it may arise from the differences in crystal symmetry; 2H1 adopts a P1 space group whereas the space group for 3E5 IgG₃ is P2₁2₁2₁, which results in different intermolecular contacts and stresses on the flexible elbow region, secondly, the differences in the Fab amino acid sequences may collectively affect the elbow angles resulting in different conformations, and third, the interplay of both mechanisms may result in the difference we observed. Furthermore, when the V regions are superimposed, we calculated the backbone rms deviations (rmsd) at the residue level for the two Fabs (Fig. 4B–D). It is important to note that the 2H1 structure is bound to Ag, PA1, while our IgG₃ crystal structure was not bound to an Ag. Residues in the structural CDRs with an rmsd above 1 Å were ^HAsp54 and ^LSer25, ^LSer26, ^LGln27, ^LSer32, ^LArg59, ^LVal99 and ^LPro100. CDR ^LSer26 is implicated in Ig peptidase activity, but a digestion study shows that 2H1 IgG₁ is unable to cleave a twelve amino acid peptide, P1 that is hydrolyzed at 37 ° C by all 3E5 IgGs except IgG₃ (Janda et al., 2012).

3.4. Molecular dynamics simulation and modeling the 3E5 IgG₃-Ag binding interface

Attempts to crystallize 3E5 IgG₃ in complex with its cognate Ag (peptide P1) were unsuccessful, though the P1 peptide was present in the supernatant for the crystallization. To gain insights into possible 3E5 IgG₃ + P1 binding mechanisms we have mutated all amino acids that distinguish the 2H1 IgG₁ and 3E5 IgG₃ Fabs *in silico*, to convert the 2H1 IgG₁ Fab structure into that of 3E5 IgG₃. The 2H1 IgG₁ (PDB: 2H1P) crystal structure was solved with and without a peptide antigen, PA1, which is a mimetic for the native polysaccharide Ag. PA1 has a similar amino acid sequence (GLQYTPSWMLVG) to P1 (SPNQHTPPWMLK). Consequently, we also mutated both 2H1 PA1 *in silico* using CHIMERA to achieve a model for 3E5 IgG₃ + P1 binding. Utilizing AMBER 11 (Case et al., 2010) we ran a 10 ns all atoms molecular dynamics (MD) simulation followed by initial minimization and equilibration steps (see Section 2.7). Along the MD trajectory the minimum energy frame was chosen and analyzed. Interestingly, upon mutating both the V and C regions of 2H1 IgG₁ to 3E5 IgG₃ (3E5 IgG₃*), the elbow angle of the minimum energy structure assumed a value (11.6°) closer to that of the native 3E5 IgG₃ structure (36.6°) (Fig. 5A) than 2H1 IgG₁ (-11.4°) (Fig. 4A). 3E5 IgG₃* + P1 adopts a similar binding interface to 2H1 IgG₁ + PA1 (Fig. 5B). The binding interactions involve hydrophobic and uncharged amino acids, with the exception of three positively charged amino acids (^HArg99, ^LHis31, ^LHis98), which resulted in several hydrogen bonds between P1 and the IgG₃ CDRs (Fig. 5C). The CDR residues making H-bond contacts with P1 were ^HArg99, ^LAsp1, ^LVal30, ^LSer96, ^LThr97 and ^LVal99. Two of these residues are also implicated in making H-bonds in the 2H1 + PA1 structure, ^LSer96, ^LThr97 (Young et al., 1997). The total energy values over the MD trajectory and the variation of elbow angle is shown in Fig. 5D: the system reached a minimum within the first couple of ns of the simulation and the elbow angle samples values between 0° and 20°. The variation of energy

is in the order of thermal fluctuation, and it is reasonable that the angular fluctuation of the elbow is of similar origin. Furthermore, the comparison of binding pockets created by flanking CDR loops revealed subtle but important differences. The binding pockets for IgG₃* and 2H1 are larger than that of IgG₃ (Fig. 6A–C). The volume difference maps of IgG₃ and IgG₃* show that the CDR loops, L1, L3, H1, H2 and H3 are shifted outwards creating a larger binding pocket surface to accommodate P1 (Fig. 6D and E). In IgG₃*, P1 has a tight fit into the cavity created by CDR loops; specifically the C-terminal end of P1 is relatively rigid with respect to the N-terminal end (Fig. 5C).

4. Discussion and conclusions

Our results provide the first direct comparison of the four murine IgG isotypes in solution. The most interesting result was the degree to which IgG₃ differs from the other IgGs. Ig shapes deduced from SAXS analysis revealed that the 3E5 IgGs had an elongated Y-shape, with a radius of gyration of 53.5–57.8 Å. The calculated R_g values were within the range of previous reports on intact IgG solution structures (Abe et al., 2010; Ashish et al., 2010; Gregory et al., 1987; Igarashi et al., 1990; Kilar et al., 1985; Lu et al., 2006; Rathore et al., 2011). The 3E5 IgG₃ isotype had both the largest R_g value and the greatest maximum molecular dimension. IgG₃ may be an outlier because of the single disulfide bond within its hinge, as compared to the three or four disulfide bonds found within the hinges of the other isotypes. Having a single disulfide contact within the hinge can allow for more Fab and Fc rotational freedom, thus creating a more flexible/extended molecule with larger R_g and D_{max} values. When comparing the Fab–Fab and Fab–Fc angles among the isotypes, major differences were discerned between IgG₁ and IgG₃ Fab–Fc angles in our models. Again, these angles were within the range of previously described angles for IgG molecules (Saphire et al., 2002). The IgG₁ Fab–Fc angle was 146°, 34° less than that of the IgG_{2a} and IgG_{2b} models, which were very similar. The IgG₃ Fab–Fc angle was 106°, 74° less than that of the IgG_{2a} and IgG_{2b} models. On the other hand, IgG_{2a} and IgG_{2b}, being the most homologous, assume the same Fc–Fab angles and similar Fab–Fab angles. Again, the 3E5 IgG₃ SAXS model had the most significant differences relative to the other isotypes, and this may also be relevant in the context of Fc receptor (FcR) binding. The murine IgG₃ Fc region is unable to interact with known FcRs recognized by other IgGs: IgG_{2a} has highest affinity for FcγRI and FcγRIV, IgG₁ and IgG_{2b} for FcγRIIβ and IgG_{2a} and IgG_{2b} for FcγRIIIα (Ravetch and Kinet, 1991). In fact, Saylor et al. recently identified that murine IgG₃ is able to promote fungal internalization in the absence of all known phagocytic receptors (Saylor et al., 2010).

The fitting of X-ray structures into the *ab initio* SAXS models revealed that the Fc domains had the largest volumes. The remaining two smaller volume arms belong to the Fab domains in each model. The X-ray Fc structure orientations are similar for all the isotypes shown (no structure is available for the IgG₃ Fc), but the Fabs are rotated in relatively different orientations. The general orientations and fit are supported by previous X-ray structures of intact IgG molecules (Saphire et al., 2002). The best-fit models done by CHIMERA are not fully definitive due to the limitations intrinsic to the *ab initio* maps calculated from SAXS data. Though the differences in Fab–Fab angles are minimal among the isotypes, the differences in Fab–Fc angles and Fab orientations with respect to the Fc domains are significantly different. These differences can be the consequences of the differences in C regions. It is known that Fab sequences affect elbow angles, *e.g.* Fabs with λ light chains sample a wide range of elbow angles and the mean elbow angles are different than the Fabs with κ light chains, which sample a narrow angular space (Stanfield et al., 2006). Thus it is possible that the differences in global solution structures of intact IgG isotypes are a consequence of different C regions. In this regard, C regions affect the functional behavior

of intact IgGs by altering their paratopes (Janda et al., 2012). It is also possible that the global structural differences imposed by C regions may affect antibody function.

In mice the immune response to bacterial polysaccharide antigens is preferential for the IgG₃ isotype (Perlmutter et al., 1978; Slack et al., 1980), IgG_{2a} is dominant in response to viral antigens (Coutelier et al., 1987) and IgG₁ is most commonly elicited in response to protein antigens (Rosenberg, 1982). It is notable that IgG₃ had the most distinct solution structure among all isotypes with the Fab–Fc angle of $106 \pm 7^\circ$, creating the largest bend with highest asymmetry among the IgG isotypes and thus different Fc domain solvent exposure. This unique exposure of the Fc domain may facilitate an immune response optimal for interacting with the types of repeating epitopes found in polysaccharide antigens. In addition, it may also be the reason for the aggregation propensity seen for the IgG₃ isotype; aggregation occurs in solution, suggesting that these molecules interact to increase avidity even before Ag binding. IgG₃ antibodies can bind cooperatively to multivalent antigens whereas V region identical IgG₁ and IgG₂ do not exhibit cooperative binding in the same conditions (Greenspan and Cooper, 1993; Greenspan et al., 1989).

The 3E5 IgG₃ Fab crystal structure revealed a predominantly hydrophobic solvent-exposed surface and gave insight into the structure between the V and adjacent C region (CH1). Comparing the 3E5 IgG₃ Fab structure to that of 2H1 IgG₁ Fab (Young et al., 1997) surprisingly revealed that both their V regions and C regions had nearly identical tertiary structures when either was aligned, even though five of their CDR residues differ. The major difference between the two Fabs was the elbow angle: angle between their V and CH1 regions. The IgG₃ Fab had a much greater elbow angle than that of the 2H1 Fab. The difference in this elbow angle must be due to the combined action of twelve amino acid residues that differ among the V regions, and thirty-one that differ between the IgG₁ and IgG₃ CH1 domains. Furthermore, Young et al. (1997) describe the unbound form of the 2H1 structure (coordinates not deposited). The residues they describe as being significantly different between the PA1-bound and unbound forms, are not the same residues that differ between the 3E5 IgG₃ Fab and the 2H1 Fab. We note however, the limitations of this comparison which involves two crystal structures with different space groups, and a 12-amino acid sequence difference where one is Ag-bound and the other Ag-unbound.

To gain insight into the effects of residues on the folding of the 2H1 and 3E5 Fab structures and to obtain a possible Ag binding model for 3E5 IgG₃, we performed *in silico* mutations and all atom molecular dynamic simulations with explicit solvent. We mutated *in silico* the 2H1 + PA1 structure to be chemically identical to the 3E5 IgG₃ Fab + P1. The minimum energy structure of the MD trajectory, 3E5 IgG₃* + P1, resulted in a structure almost identical to our 3E5 IgG₃ Fab crystal structure. Thus, the elbow angle shifted from -11.4° to $+11.6^\circ$, which is closer to the angle of the 3E5 IgG₃ Fab elbow. This result was surprising but supports studies done in our lab which show that the 3E5 IgG₃ and 2H1 IgG₁ both are unable to catalyze the P1 peptide, while the other 3E5 isotypes are able (Janda et al., 2012). The MD simulations are done with explicit solvent models and lack the neighboring Ab molecules, thus there is no stress arising from the intermolecular/crystal contacts. In addition, in the P1 bound structure, IgG₃*, the CDR loops that encompass the binding pocket are all slightly shifted outwards creating a larger space that enables P1 binding. The binding pocket of IgG₃* is thus larger than that of IgG₃ and noticeably different than that of 2H1 as a consequence of differing CDR residues and the presence of PA1. Thus the MD model for IgG₃ + P1 sheds light onto the P1 binding and the comparison of the binding pockets of IgG₃* and 2H1 hints toward paratope differences.

Furthermore, recent studies analyzing the catalysis of P1 by the 3E5 family of mAbs (Janda et al., 2012) revealed that both the 3E5 IgG₃ as well as 2H1 IgG₁ are unable to cleave the P1

peptide at 37 °C. Analysis of the putative Asp1-Ser26-His98 “catalytic triad” in the CDR regions of the 2H1 IgG₁ Fab, 3E5 IgG₃ Fab and 3E5 IgG₃* Fab, resulted in finding that distances between these atoms were all greater than 5 Å; too large for a hydrolysis reaction to be carried out, complementing the findings of Janda et al. (2012). Thus, these two Fab molecules may have more similarities in their paratopes than the 3E5 IgG₃ does with its fellow 3E5 isotypes, supporting the SAXS findings in that the crosstalk between V and C regions results in differences in paratope, peptide catalysis potential as well as global structural shape.

In a recent study, Sela-Culang et al. compared bound and free Abs and reported conformational changes related to Ag binding. Observed structural changes were found to occur far from the Ag binding site, suggesting allosteric communication between remote sites of Ags (Sela-Culang et al., 2012). In this study we complement those findings from a different view; the IgG isotypes expressing different C and identical V regions present significantly different solution structures. Our studies provide new information about IgG solution conformations, constraints imposed by differing C regions and functional relevance of global structures. The 3E5 isotype study reveals that the same V region interacts differently with different C regions, to the extent that overall shapes of the molecules are significantly different. Among all IgGs studied to date no trends or generalizations can be made within an Ig class despite similarities in R_g values, D_{max} values, hinge angles and Fab/Fab and Fab/Fc angles. Since most of these IgGs have different V regions, variability in structure suggests that C regions do not solely control their overall shapes. The solution and X-ray conformations are likely due to cross-domain interrelationship between different V regions and the constraints they put on that C region and *vice versa*, since small changes to a few Fab residues are enough to dramatically change the overall shape of an Fab. Improved Ab engineering for research and therapeutics will likely arise from identifying which particular regions and residues are constraining the others. This type of identification requires high-resolution structures of intact antibodies and accurate computational algorithms.

In summary, our studies highlight the extent to which the murine IgG₃ isotype is different from the other IgG subclasses. IgG₃ is clearly a physiologically important isotype given that IgG₃-deficient mice exhibit increased susceptibility to encapsulated pathogens and reduced susceptibility to autoimmune diseases (Greenspan et al., 2012; McLay et al., 2002). Furthermore, IgG₃ interacts with a different receptor than the other IgG subclasses (Saylor et al., 2010). Our studies also provide more evidence for the notion that Ig V and C domains influence each other structurally both on the intact as well as Fab scales. Finally, the diversity of immunoglobulin structures in the literature suggest the need for additional studies comparing structures both across and within isotypes to ascertain the degree to which allosteric effects from the combination of different V and C domains influence overall antibody structures.

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Fig. 1. Small angle X-ray scattering and distance distribution profiles. SAXS profiles of IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ are shown in A, B, C and D respectively, where q is in ($\text{\AA}^{-1}$). The inserts show the Guinier plots and obtained R_g values. The low- q near-linear regions in scattering profiles (see text) are selected by GNOM to calculate distance distribution profiles (P_R) of IgG₁, IgG_{2a}, IgG_{2b} and IgG₃, shown in E, F, G, H respectively, where R is in ($\text{\AA}$), R_g of each Ig, calculated by integrating P_R , are shown.

Fig. 2. Calculated *ab-initio* 3E5 IgG models. The Fab–Fab and Fc–Fab angles are depicted. The high-resolution X-ray structures with best fitting orientations are shown in ribbon diagrams. The PDBs of X-ray structures are 1IGY (in IgG₁ map), 1IGT (in IgG_{2a} map), 1MAM (Fab) and 2RGS (Fc) (in IgG_{2b} map) and 4HDI (in IgG₃ map). χ^2 values and a plot of the model fit to the experimental $I(q)$ vs q for each isotype are shown for the best models on the right panel.

Fig. 3. 3E5 IgG₃ Fab structure (PDB ID: 4HDI). (A) 2.45 Å resolution crystal structure of IgG₃ Fab. (B) IgG₃ Fab exists as a dimer in asymmetric unit (AU). (C) Antigen binding pocket comprised of CDR loops. (D) Electrostatic surface (Coulombic) of Ag binding pocket. (E) Ag binding pocket residues plotted as surfaces and colored by their solvent exposed areas, cyan – most solvent exposed; red – least solvent exposed.

Fig. 4. Comparison of 3E5 IgG₃ (PDB: 4HDI) (orange) and 2H1 IgG₁ (PDB: 2H1P) (cyan) X-ray crystal structures. (A) Two structures are overlaid using their Fc regions as references. The elbow angles are different (36.6° for 3E5 IgG₃ and 11.4° for 2H1 IgG₁). (B) Overlay of the variable regions (H chain on top, L chain on bottom). (C) The rms deviations between V region residues, the deviations are higher for the light-chain residues. (D) Color coding of the rms deviations plotted on to the IgG₃ ribbon structure (H chain on top, L chain on bottom).

Fig. 5. IgG₃-P1 binding model generated by MD simulation. 2H1 IgG₁ + PA1 complex is mutated (both V and C regions) to model 3E5 IgG₃ + P1 binding. (A) Overlay of 3E5 IgG₃ and mutant (3E5 IgG₃ * + P1). The difference in elbow angles is decreased when compared to the difference between 3E5 IgG₃ and 2H1 IgG₁. The overlaid image is sampled at the same orientation as in Fig. 4. (B) A surface map showing the binding interface, IgG₃ * is in blue, P1 in light pink. Red patches depict contacting surfaces. Residues of P1 involved in binding are colored red in the chain trace. Residues toward the N-terminal have higher degree of flexibility with fewer contacts than the others. (C) Interactions between IgG₃ * and P1 are mostly comprised of hydrophobic residues. (D) Graph showing the total potential energy and hinge angle variations over 10 ns MD trajectory. The first 0.1 ns is the time allocated for heating, density equilibration and equilibration steps prior to which a 2000 steps minimization is performed, the trajectory for minimization is not recorded and not shown in the graph. The vertical red line and the circle mark the point of minimum energy structure.

Fig. 6.

A comparison of the 2H1 and 3E5 binding pockets. The binding pockets encompassed by CDR loops are shown for (A) IgG₃, (B) IgG₃ * and (C) 2H1. The binding pocket is enlarged in IgG₃ * when compared to IgG₃, in order to accommodate P1. (D) The volume difference map of IgG₃ - IgG₃ *, the binding pocket area is depicted by a dashed loop. (E) The volume difference map of IgG₃ * - IgG₃. Due to the outward movements of CDRs in IgG₃ * the area is enlarged.

Table 1

X-ray statistics of data collection and refinement. Numbers in parenthesis are values for the highest resolution shell.

Data collection	
Wavelength (Å)	0.9790
Space group	<i>P2₁2₁2₁</i>
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	67.070, 98.289, 142.363
α , β , γ (°)	90, 90, 90
Resolution (Å)	50.0–2.45 (2.54–2.45)
Unique reflections	35,380
<i>I</i> / σ	20.8 (3.0)
Completeness (%)	99.9 (100)
Redundancy	6.5 (6.7)
<i>R</i> _{merge}	0.082 (0.535)
Refinement	
Resolution (Å)	43.76–2.45 (2.51–2.45)
Number of reflections	33,544
<i>R</i> _{work}	0.208 (0.307)
<i>R</i> _{free}	0.264 (0.384)
Number of atoms	
Protein	6677
Water	65
<i>B</i> factors (Å ²)	
Protein	44.64
Water	49.99
RMSD from ideal geometry	
Bond length (Å)	0.010
Bond angles (°)	1.43