Demonstration by pulsed neutron scattering that the arrangement of the Fab and Fc fragments in the overall structures of bovine IgG1 and IgG2 in solution is similar

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The bovine IgG1 and IgG2 isotypes exhibit large differences in effector functions. To examine the structural basis for this, the 12-domain structures of IgG1 and IgG2 were investigated by pulsed neutron scattering using a recently developed camera LOQ. This method reports on the average relative disposition in solution of the Fab and Fc fragments in IgG. The radii of gyration (R_G) were found to be similar at 5.64 and 5.71 nm for IgG1 and IgG2 respectively in 100% $^2\text{H}_2\text{O}$ buffers. The two cross-sectional radii of gyration (R_{XS}) were also similar at 2.38–2.41 and 0.98–1.02 nm. Similar values were obtained for porcine IgG. Both bovine IgG1 and IgG2 possess similar overall solution structures, despite sequence differences at the hinge region at the centre of their structures. An automated computer survey of possible IgG structures was developed, in which co-ordinates for the two Fab fragments were displaced in a two-dimensional plane relative to those of the Fc fragment in 0.25 nm

steps. The scattering curves calculated from these structures were found to be sensitive to relative displacements of the three fragments, but not on their rotational orientation about their longest axes. Good agreement with the solution scattering data was obtained with a planar IgG model in which the C-terminus of the C_H1 domain of Fab was 3.6 nm from the N-terminus of Fc in both IgG1 and IgG2, with a precision of 0.7 nm. Energy refinement showed that this spatial separation is compatible with the hinge sequences of bovine IgG1 and IgG2. The results show that multidomain protein structures can be modelled using LOQ data, and that a long hinge sequence does not necessarily reflect a large distance between Fab and Fc. The steric accessibility of Fc sites for interactions with cell-surface Fc receptors and C1q of complement is shown to be generally similar for IgG1 and IgG2, and the difference in effector function between IgG1 and IgG2 is probably based on deletions in the IgG2 hinge sequence.

INTRODUCTION

IgG plays a central role in a wide variety of immunological processes by mediating the link between antigen and effector [1]. Diversity of the variable domains in the Fab fragment of IgG ensures the recognition of a vast array of antigens, and determinants located in the Fc fragment allow for interaction with receptors. The two Fab fragments (V_H , V_L , C_H1 and C_L domains) and one Fc fragment (two C_H2 and two C_H3 domains) in IgG are linked by a disulphide-linked polypeptide hinge. An association of the hinge region– C_H2 domain of IgG with effector sites in IgG has been described [2]. In order to understand the differences in effector function presented by different IgG isotypes, knowledge is required of the structural features of the hinge regions in these isotypes.

A major functional difference between IgG isotypes occurs in ruminants. Only IgG1 is selectively transported from blood plasma into colostrum by specific receptors which appear on the surface of mammary epithelial cells in the period leading up to parturition [3–5]. Although the identity of the amino acid residues of bovine IgG1 that interact with the receptor is at present unknown, they are known to reside on the heavy chain and are likely to be located near the N-terminus of the C_H2 domain [6]. Receptor specificity for IgG1 and not IgG2 could result from a binding site at the hinge region– C_H2 domain in IgG1 that is not present in IgG2, from different hinge conformations in IgG1 and

IgG2, or from steric obstruction of the Fc site by the Fab fragments in IgG2. Sequencing and structure-prediction studies have accordingly been carried out for the heavy chains of bovine and ovine IgG1 and IgG2 [7–10]. Exploratory molecular modelling resulted in IgG1 models with extended hinge structures and little restraint on Fab–Fc flexibility. Bovine and ovine IgG2 have a deletion in the hinge sequence with loss of the determinant motif for the high-affinity Fc γ RI receptor. Predicted models for IgG2 were accordingly restricted to a short hinge, and steric hindrance of effector function was considered to be likely.

Structural data on hinge regions are now required. In general, segmental flexibility between the Fab and Fc fragment hinders crystal structure determinations for IgG except in restricted cases where the hinge is either non-existent (the hinge-deleted human IgG1 Dob and IgG1 Mcg structures [11,12]) or is sufficiently inflexible to permit crystal formation (mouse IgG2a [13]). Solution scattering [14,15] is a useful technique to study domain arrangements in multidomain proteins under conditions close to physiological. If atomic structures are available for individual domains, it is possible to evaluate domain arrangements that are compatible with the scattering curves, as these are fully calculable from crystal structures [16–18]. To assess these IgG structures in a systematic manner, a new method of automated scanning of possible structures has been evaluated, using neutron data from a recently developed scattering camera LOQ which permits the simultaneous measurement of a full scattering curve $I(Q)$ [19].

Abbreviations used: R_G , overall radius of gyration; R_{XS} , radius of gyration of the cross-section.

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Neutron scattering has advantages in that possible radiation damage effects encountered with X-rays are absent, and the high solvent contrast obtained by data collection in 100 % $^2\text{H}_2\text{O}$ minimizes the systematic errors in curve fitting caused by internal density inhomogeneities. We show that a moderately extended hinge region with correct stereochemistry is able to account for the solution structures of bovine IgG1 and IgG2.

MATERIALS AND METHODS

Preparation of bovine and porcine IgG

IgG was precipitated from bovine or porcine serum by the addition of 0.5 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. The precipitated immunoglobulin fraction was dissolved and equilibrated with 0.01 M phosphate buffer, pH 8.0. The bovine IgG1 and IgG2 isotypes were separated by ion-exchange column chromatography on DEAE-cellulose (Sigma) equilibrated with the 0.01 M phosphate buffer, pH 8.0. The IgG2 isotype was not adsorbed, and the bound IgG1 isotype was eluted with 0.044 M phosphate, pH 6.97 [20]. Pig IgG equilibrated with 0.0175 M phosphate, pH 6.5, was purified on DEAE-cellulose equilibrated with the same buffer solution. The non-adsorbed fraction comprised pig IgG. Fractions were pooled on the basis of their immunoelectrophoretic patterns, using anti-(whole serum) as developing reagent, yielding preparations of IgG1 and IgG2 having no detectable contamination with the other isotype. Samples were concentrated in Centriprep 10 concentrators (Amicon). Preparations were cleared of aggregates shortly before neutron-scattering-data collection by gel filtration through Superose 6 using an FPLC system (Pharmacia). Proteins were dialysed at 6 °C with four changes of buffer over at least 36 h into PBS (12 mM sodium/potassium phosphate, 140 mM NaCl, pH 7.4) in 99.8 % $^2\text{H}_2\text{O}$ to ensure the fullest possible exchange of H_2O and exchangeable protons. The full composition was approximated by use of the crystallographically observed sequences for human Fab NEW and Fc KOL with the amino acid sequences of the hinge region of bovine IgG1 and IgG2 [7]. IgG concentrations for neutron M_r calculations were calculated from absorbance measurements at 280 nm using an absorption coefficient (1 %, 1 cm) of 13.5. This is consistent with values of 13.7 and 14.1 calculated from the estimated bovine IgG1 and IgG2 compositions [21].

Pulsed-neutron data collection at ISIS

Neutron-scattering data were obtained in three different beam sessions on the LOQ instrument at the pulsed-neutron source ISIS at the Rutherford–Appleton Laboratory, Didcot, U.K. [19]. The pulsed neutron beam was derived from proton beam currents of 90–170 μA . Monochromatization was achieved using time-of-flight techniques. A ^3He ORDELA wire detector recorded scattered intensities at a sample-to-detector distance of 4.3 m. Samples and their $^2\text{H}_2\text{O}$ buffers were measured in 2 mm-thick rectangular Hellma cells thermostatically controlled at 15 °C. Some 2×10^6 – 4×10^6 monitor counts were accumulated in runs lasting 40–80 min each for protein concentrations between 3 and 12 mg/ml. Intensities were normalized relative to the scattering from a partially deuterated polystyrene standard [22]. All sample and buffer transmissions were measured. Data reduction utilized the ISIS software package COLETTE [23], in which intensities were binned into individual diffraction patterns to correspond to wavelength ranges between 0.22 and 1.00 nm, and corrected for a linear wavelength-dependence of sample transmissions. The merger of these patterns gave the full scattering curve $I(Q)$ in a Q range of 0.05–2.2 nm^{-1} ($Q = 4\pi \sin \theta/\lambda$; 2θ = scattering angle;

λ = wavelength). The Q range was based on 0.04 % logarithmic increments, which was optimal for Guinier R_g and $I(0)$ analyses at low Q and for improved signal-to-noise ratios at large Q .

Analysis of reduced neutron data

The radius of gyration (R_g) measures structural elongation if the internal variation of scattering densities within the macromolecule has no effect. Guinier analyses at small Q give the R_g and the forward scattering at zero scattering angle $I(0)$ [14]:

$$\ln I(Q) = \ln I(0) - R_g^2 Q^2/3$$

The $I(0)/c$ values (c = sample concentration) for samples measured in the same buffer give the protein M_r [24]. For elongated structures, the radius of gyration of the cross-section (R_{xs}) and the cross-sectional intensity at zero angle $[I(Q)Q]_{Q \rightarrow 0}$ is obtained from:

$$\ln [I(Q)Q] = \ln [I(Q)Q]_{Q \rightarrow 0} - R_{xs}^2 Q^2/2$$

For immunoglobulins, the cross-sectional plot exhibits two regions, a steeper innermost one and a flatter outermost one [25], denoted by R_{xs-1} and R_{xs-2} respectively. The R_g and R_{xs} analyses led to the triaxial dimensions of the macromolecule. If the structure can be represented by an elliptical cylinder, $L = [12(R_g^2 - R_{xs}^2)]^{1/2}$, where L is its length [14]. Alternatively, L is given by $\pi I(0)/[I(Q)Q]_{Q \rightarrow 0}$ [26]. Data analyses employed an interactive graphics program SCTPL4 (A. S. Nealis and S. J. Perkins, unpublished software) on a Silicon Graphics 4D35S workstation.

Indirect transformation of the scattering curve $I(Q)$ to give the distance distribution function $P(r)$ was carried out using ITP-91 [27].

$$P(r) = \frac{1}{2\pi^2} \int_0^\infty I(Q) Q r \sin(Qr) dQ$$

For a uniform scattering density, $P(r)$ corresponds to the distribution of distances r between any two-volume elements within the macromolecule. This leads to an alternative calculation of R_g and $I(0)$ that is based on the full scattering curve, and gives the largest dimension of the macromolecule L . For IgG1 and IgG2, the neutron $I(Q)$ contained 75 data points extending out to 2.04 nm^{-1} and was fitted with eight splines with an assumed maximum distance D_{max} set at 30 nm. $P(r)$ was defined by 101 points. Criteria for the correct transformation parameters were that $P(r)$ should exhibit positive values, the ITP-91 R_g should agree with the Guinier R_g , $P(r)$ should be zero when r is zero, and $P(r)$ should be stable and reproducible when the number of splines and D_{max} is varied over a reasonable range. At large r , L was determined when $P(r)$ became zero, although errors in L can be significant as a result of the low intensity of $P(r)$. $P(r)$ curves were also calculated using GNOM [28,29]. This utilizes a different regularization procedure with an automatic choice of a transformation parameter α to stabilize the calculation of $P(r)$. A range of D_{max} values was tested, and the final choice of D_{max} was based on the stability of $P(r)$ as D_{max} was increased beyond the estimated macromolecular length.

Debye sphere modelling of bovine IgG1 and IgG2

Scattering-curve calculations were initiated using the co-ordinates for the bovine IgG1 and IgG2 models of Symons et al. [7]. The models were created from crystal structures for human IgG Fab NEW and human IgG1 Fc KOL fragments [30,31] (Brookhaven codes 3fab and 1fc1). The hinge was created using FRODO, in which each hinge residue was geometrically

optimized one-by-one for bond lengths and angles and side-chain collisions. For the present study, further IgG1 and IgG2 models were created using Insight II V2.3.0 molecular modelling software (Biosym Technologies Inc.) on a Silicon Graphics Indigo XS24 workstation. To generate many models, a Biosym Command Language (BCL) macro was written to select and move the two Fab fragments as distinct objects relative to the hinge and Fc fragment before storage of the resulting structure. Carbohydrate was added to Fc in a further BCL macro using the residues found in the Fc KOL co-ordinates. Another BCL macro was used to rotate the Fc fragment about its twofold axis of symmetry and then add carbohydrate. Models were checked for correct domain movements using the Axes command. Visual checks and the Bump command ensured that no van der Waals overlaps occurred between the Fab and Fc fragments.

To convert the atomic co-ordinates into spheres, each full IgG co-ordinate model was placed in a three-dimensional grid of cubes of side 0.61 nm. A cube was included in the sphere model if these contained sufficient co-ordinates above a cut-off value, which was determined by varying this until the total volume of the cubes agreed to within 1% difference of the dry protein and carbohydrate volume. This volume was computed from the amino acid and carbohydrate residues present in the co-ordinates [21,32]. The cube size is much less than the nominal resolution of $2\pi/Q_{\max}$ of the scattering curves, which is 4.1 nm for $Q_{\max} = 1.55 \text{ nm}^{-1}$ in this study. Overlapping spheres of volume equal to each cube were placed at the centre of each cube. The scattering curve $I(Q)$ was calculated from 773–797 spheres according to Debye's Law adapted to spheres:

$$I(Q) = \sum I_j(Q) + 2 \sum \sum B_j(Q) B_k(Q) \sin(Qr_{jk})/Qr_{jk}$$

for N spheres of form factor $B(Q)$ and with $j, k = 1$ to N and $j \neq k$ [14]. Calculations of the R_g of the model, and the goodness-of-fit parameter R between the calculated and experimental curve out to Q of 1.6 nm^{-1} followed refs. [16,33]. Corrections were empirically applied for neutron wavelength spread and beam divergence using the procedure previously used for Instrument D17 at the Institut-Laue-Langevin, Grenoble, France [33,34]. On Instrument LOQ, the wavelength resolution increases as λ increases. The effect of this on the experimental data could not be detected when the curves were calculated using wavelength binning ranges of 0.22–1.0 nm, 0.22–0.4 nm, 0.4–0.6 nm, 0.6–0.8 nm and 0.8–1.0 nm. This showed that an averaged correction for wavelength spread and divergence could be applied to the calculated curves. After a range of wavelength spread and beam divergence parameters had been tested, values of 16% spread for a nominal λ of 1.0 nm and a beam divergence of 0.016 radians were selected. These are fortuitously the same as those used to fit D17 data.

RESULTS AND DISCUSSION

Neutron-scattering data on bovine and porcine IgG

Neutron-scattering data for bovine IgG1 and IgG2 were analysed in three distinct Q ranges in order to obtain R_g , R_{xs-1} and R_{xs-2} . Figure 1 shows that these parameters were obtained from linear Guinier plots in satisfactory QR_g and QR_{xs} ranges. Although some Guinier analyses were affected by noise, the data were reproducible in two sessions with different preparations. No concentration-dependence was observed between 3 and 12 mg/ml. The $I(0)/c$ values reflect the M_r of IgG1 and IgG2. The $I(0)/c$ values were identical within experimental error at relative values of 0.182 and 0.180 (S.D. ± 0.006) for IgG1 and IgG2 respectively. This agreement is as expected from the

sequences and their similar contents of Trp, Tyr and Cys residues which affect the calculation of c [7,21]. Purification by gel-filtration methods ensures that the scattering curves correspond to monodisperse IgG preparations. As the R_g values (Table 1) are, within experimental error, identical at 5.64 and 5.71 nm respectively, and the two R_{xs} values are similar at 2.38–2.41 nm and 0.98–1.02 nm, no significant structural differences between the IgG1 and IgG2 isotypes can be inferred. This is consistent with sedimentation coefficients which are similar for both IgG1 and IgG2 [5].

The R_g/R_o ratio indicates the anisotropy (or the degree of elongation) of the protein structure (where R_o is the R_g of the sphere with the same dry volume as the protein). The R_g/R_o ratio was determined to be 2.07 and 2.10 for IgG1 and IgG2 respectively. Both are larger than the value of 1.28 ± 0.10 for 53 standard globular proteins [15]. The solution structure of IgG1 and IgG2 is extended, as expected from crystallography and microscopy data [11–13,36]. The length L of the longest macromolecular axis was determined to be 17.7–18.2 nm from the Guinier analyses (Table 1). The full scattering curves $I(Q)$ of IgG1 and IgG2 were very similar (not shown). Calculations of the distance distribution function $P(r)$ from $I(Q)$ showed (Figure 2) that the overall structures of IgG1 and IgG2 were similar. The maximum in the $P(r)$ curve occurs at 5.4–5.7 nm. The intersection of $P(r)$ with the zero axis at high r gives the maximum dimension L of IgG1 and IgG2 to be 15.6 and 16.2 (S.D. ± 2.1) nm.

As a control of consistency, the bovine IgG data were compared with previous neutron scattering on IgG samples. Preparations of porcine anti-dinitrophenyl hapten IgG had resulted in noticeably higher R_g values of 6.74 ± 0.08 and 6.16 ± 0.07 nm [37], and another one of 6.48 nm [38] when compared with Table 1. While the Guinier plots used to determine the R_g values were not shown in [37,38], the higher R_g values cannot be attributed to the use of a different Guinier Q range for fits. It is possible that the higher R_g values are due to the inability of centrifugation at 6000 g to clear aggregated material. Human myeloma IgG also gave a higher neutron R_g of 7.24 ± 0.13 nm [39]. The difference may be explained on the basis of dimer formation which was concluded from non-linear Guinier plots. To resolve the differences with previous studies, we examined porcine IgG that was purified by gel filtration to remove aggregates. This contains at least five subclasses with hinge sequences of lengths similar to those of bovine IgG, although, until the germline is mapped, other porcine isotypes may yet be identified. In linear Guinier plots (not shown), porcine IgG was found to have an R_g of 5.28 nm, and R_{xs} values of 2.27 nm and 1.22 nm (Table 1), all of which are close to those seen for bovine IgG. The $I(0)/c$ value for porcine IgG was 0.175 ± 0.004 , which shows that this is monomeric when compared with the $I(0)/c$ values of 0.180–0.182 for bovine IgG. It is of interest to note that a neutron R_g value of 5.75 nm for rabbit anti-dinitrophenyl hapten IgG was determined [35], which is close to our determinations (Table 1). Rabbit IgG was also shown to be monomeric from $\sqrt{I(0)/c}$ plots. It would appear that the use of gel filtration to remove IgG aggregates, combined with satisfactory $I(0)/c$ analyses, will lead to consistent R_g determinations for IgG. As it is the physiological function of IgG to aggregate to form immune complexes, such precautions are considered to be important for solution studies of antibody structures.

Scattering data at larger Q are less affected by oligomer formation. Previous R_{xs} determinations in [35,37–39] agree to within 0.2 nm (at most) of the present values for bovine IgG1 and IgG2 (Table 1). The neutron R_{xs} values are slightly reduced by about 0.2 nm when compared with X-ray R_{xs} data for the human IgG subclasses [40]. This is attributable to the effects of

Figure 1 Neutron Guinier R_G and R_{XS} plots for bovine IgG1 in 100% $^2\text{H}_2\text{O}$

IgG1 was measured at concentrations of 12.1 and 8.1 mg/ml. The filled circles between the QR_G and QR_{XS} ranges as arrowed show the data points used to determine R_G and R_{XS} values, using the Q ranges summarized in Table 1. Data outside these ranges are denoted by open circles. Error bars correspond to the statistical errors of LOQ data collection.

Table 1 Neutron Guinier analyses of IgG in $^2\text{H}_2\text{O}$ buffer

The R_G and R_{XS} values are means $\pm$ S.D. of the indicated number of separate measurements made between 3 and 12 mg/ml, where the S.D.s correspond to those from the Guinier fits. The Q range used for fitting the R_G data was 0.10–0.19 nm^{-1} , and those for the R_{XS-1} and R_{XS-2} data were 0.30–0.46 nm^{-1} and 0.56–1.04 nm^{-1} respectively. The L values correspond to the longest dimension of the macromolecule calculated from either R_G (assuming an elliptical cylinder: upper value) or $I(0)$ (assuming an elongated structure: lower value). The data for rabbit IgG are from Gilmour et al. [35], and were collected on Instrument D11 at the Institut-Laue-Langevin, Grenoble.

Protein	R_G (nm)	R_{XS-1} (nm)	R_{XS-2} (nm)	L (nm)
Bovine IgG1	5.64 ± 0.28 (4)	2.38 ± 0.09	1.02 ± 0.07	17.7 ± 1.2 17.8 ± 1.8
Bovine IgG2	5.71 ± 0.51 (3)	2.41 ± 0.12	0.98 ± 0.06	17.9 ± 2.1 18.2 ± 1.6
Porcine IgG	5.28 ± 0.45 (3)	2.27 ± 0.06	1.22 ± 0.06	16.5 ± 1.8 17.4 ± 1.1
Rabbit IgG	5.75	2.16	0.98	

(i) density inhomogeneity within IgG, where neutrons using 100% $^2\text{H}_2\text{O}$ buffers view IgG in a high negative solute–solvent contrast, whereas X-rays do so in a moderate positive contrast difference [15], and (ii) hydration of IgG1 and IgG2 that is detectable by X-ray scattering but not by neutrons [16].

Scattering curve modelling of bovine IgG1 and IgG2

The neutron data can be directly compared with predicted models for bovine IgG [7]. This is based on a method for scattering-curve simulations from spheres, starting from atomic co-ordinates. The method has been calibrated with α_1 -antitrypsin, β -trypsin and α -chymotrypsin [16,18]. Scattering models for bovine IgG1 and IgG2 were tested for three types of hinge (see Figure 6 for ovine IgG [10]). IgG1 models with the long and medium hinges gave R_G values of 5.92 and 5.39 nm respectively, and the IgG2 model with a short hinge gave an R_G value of 5.00 nm (Figure 3). The long- and medium-hinge models gave

Figure 2 Neutron distance distribution functions $P(r)$ for bovine IgG1 and IgG2

The continuous line with error bars corresponds to $P(r)$ for IgG1 using ITP-91, and the dotted line to IgG2. The dashed line corresponds to $P(r)$ for IgG1 calculated from GNOM. The maximum M in $P(r)$ occurs at 5.4–5.7 nm, and the maximum dimension was determined to be 15.6 ± 2.1 nm.

the closest agreement with the experimental R_G values of 5.64–5.71 nm (Table 1).

Comparison of the experimental and the three predicted $I(Q)$ curves showed that remodelling was required to yield good curve fits. The medium-length hinge model was used as starting point for automated curve simulations. As scattering depends on the relative arrangement of the Fab and Fc fragments, and as the hinges are not directly detectable for reason of their low mass, only relative fragment positions were considered at this stage. Automated curve-fitting calculations systematically explored the effect of displacing the Fab co-ordinates held in the same relative fixed orientation to each other in 0.25 nm steps along the vertical

Figure 3 α -Carbon plot of the Fc fragment of IgG1 and IgG2 and three models for the hinge region

The C_{H2} and C_{H3} domains in the crystal structure of human IgG1 Fc KOL are shown in thin outline together with the three hinge structures shown in bold outline [7]. The long hinge consists of two polyproline helices based on the 16-residue sequence KPSPDCPPPELPGG (IgG1), whereas the medium hinge is based on the same sequence with the replacement of each polyproline helix with two reverse turns at the C-terminus. Note that both sequences are held together by three disulphide bridges (O) near their N-terminus (N). The short hinge is based on the 11-residue sequence SKPNNQHCVRE (IgG2), and is held together by a disulphide bridge at the lower end of the hinge (O).

twofold axis of symmetry in the Fc fragment. At each vertical displacement, the effect of varying the horizontal separation of the two Fab fragments in 0.25 nm steps was explored as well. Each model was converted into spheres for curve simulations. After correction for beam wavelength and divergence (see the Materials and methods section), the predicted curve was fitted automatically to the experimental curve. The quality of the curve fit was assessed by comparison of the calculated and experimental R_G values, visual comparisons of sets of curve fits, and the minimization of R which quantifies the agreement of the curve fit over the available Q range between 0.09 and 1.55 nm⁻¹ [16]. After initial trials to assess how best to generate IgG models, about 100–200 models could be tested within hours to lead to a limited family of models that gave good curve fits with the experimental data.

The calculated curves are sensitive to the relative displacements of the Fab and Fc fragments within IgG. Displacement of the two Fab fragments in eight steps along the Fc vertical axis of symmetry (Figure 3) from -2.0 to +1.5 nm in the medium-hinge IgG model caused the R_G to increase from 4.9 to 5.9 nm. The calculated $I(Q)$ curves change noticeably in the Q ranges used for R_G , R_{XS-1} and R_{XS-2} (Figure 4a). An increase in the Fab horizontal separation from 0 to 3 nm in seven steps in the same plane caused the R_G to increase from 5.4 to 6.3 nm, with corresponding changes in the $I(Q)$ curves (Figure 4b). As the curves in Figures 4(a) and 4(b) demonstrate similar variations, it is not possible to determine a unique structure for IgG. From the 56 models thus tested, four good curve fits were obtained. The first of these correspond to an IgG model in which the Fab fragments were moved closer to the Fc fragment by a 0.5 nm vertical step. Three further fits were obtained by successive 0.5 nm vertical steps towards the Fc fragment, where each step was accompanied by an increased horizontal separation of 0.5 nm

between the two Fab fragments. The spatial distance between the Val α -carbon at the C-terminus of β -strand G in the Fab fragment and that of the Pro α -carbon at the N-terminus of the Fc fragment (see Figure 6) was 3.6 nm in the first model. This became 3.3, 3.1 and 2.9 nm in the other three models in that order.

Figure 5 shows the first of this family of allowed models. It has an R_G value of 5.31 nm which is slightly smaller than the experimental value of 5.64–5.71 nm, and shows good agreement over the full Q range. The goodness-of-fit R is 0.012 for 73 data points in the Q range of 0.09–1.55 nm⁻¹. To assess the range of allowed IgG structures, Figure 5 shows that the vertical displacement of the Fab fragments up or down by 1.5 nm is noticeable, and gave worsened R of 0.016 and 0.018 respectively. For comparison, the long-, medium- and short-hinge models of Figure 3 gave R of 0.018, 0.013 and 0.018 in that order. Rotational reorientation of the Fc fragment in the IgG model in 45° steps about its vertical axis caused minor perturbations in which R varied by 0.002 at most. Replacement of the two Fab fragments in the final model by the dimeric Fab'₂ crystal structure from human IgG1 KOL showed almost no effect on the calculated curve. These show that no information is available on Fab or Fc rotamers in the final IgG model.

Sequence analysis of the hinge region

The IgG scattering model requires a clarification of how the Fab and Fc fragments will be connected by the bovine IgG1 and IgG2 hinge sequences. This will be defined by the location of the β -strands that comprise the protein core of the Fab and Fc fragments, and also by the position of Cys residues. Multiple sequence alignment using the program MULTAL [41] and final adjustment by eye was carried out for four ruminant heavy-chain

Figure 5 Comparison of experimental and calculated curves for bovine IgG

The experimental curve is shown with statistical error bars (●). The calculated curve corrected for wavelength resolution and beam divergence is shown as the continuous line (R of 0.012). Two other models in which the two Fab fragments are displaced by 1.5 nm above and below the best-fit IgG model give rise to the simulated curves denoted by dashed lines (R of 0.016 and 0.018). The corresponding sphere model for the best curve fit is shown using spheres of diameter 0.61 nm, together with an α -carbon representation of this model. The α -carbon positions of the receptor motif LPGGP and the C1q site EKK are shown as filled circles.

IgG1 and IgG3 and mouse IgG2a that binds to the high-affinity Fc γ RI receptor in macrophages [2,10,45]. Although the statistical error of the R_g determination is ± 0.45 nm, the porcine R_g value appears slightly smaller than those for bovine IgG. This may be compared with a 'structural' hinge of 11 residues for all five porcine isotypes shown in Figure 6.

It is necessary to show that a satisfactory stereochemistry for both hinge sequences can be generated to connect Fab and Fc in the final IgG scattering model. Energy-refined hinges were generated for bovine IgG1 and IgG2 using X-PLOR [46]. For bovine IgG1, the sequence VEPKSCKPSPCDCCPPPELPGG-PSVFLFPP was used. The underlined residues in bold occur in the 3fab and 1fcl crystal structures, and 17 of the 19 residues in the centre correspond to the structural hinge in the bovine sequence. The residues in bold in the IgG scattering model with an α -carbon separation of 3.6 nm between Fab and Fc were fixed as constraints. The sequence VEPKSCSKPNNQHCVRE-PSVFLFPPKPK was used for bovine IgG2, with 12 residues from the structural hinge and the bold residues fixed. Energy refinement of the central 19 or 12 residues was carried out by a conjugate gradient minimization procedure until no further changes were seen in the resulting energy gradient. Both hinge conformations converged to a satisfactory total energy value. These were verified using PROCHECK [47] to show that the stereochemistry of the hinge was correct. This procedure gave an IgG1 model with a similar polypeptide backbone structure to that of the medium model of Figure 3, although the side chains along the hinge had improved interaction energies. The short hinge of IgG2 had a backbone structure that was now marginally straighter. The error range in the scattering models permits the IgG2 hinge to be shorter than the IgG1 hinge by up to 0.7 nm. This is derived from the spatial distance of 2.9–3.6 nm between the α -carbon atoms in the four good-fit IgG models discussed

above. Any structural differences between the two hinges that are less than 0.7 nm are too small to be resolved by scattering.

Conclusions

Differences in the bovine and ovine IgG1 and IgG2 sequences have resulted in hypotheses that a sequence deletion in the hinge and/or steric hindrance between the Fab and Fc fragments may be involved in the failure of ruminant IgG2 to interact with its mammary Fc receptor [7,10]. Here, solution scattering was used to explore possible arrangements of the Fab and Fc fragments. Even though it has been shown (Figure 4) that the scattering curves are sensitive to the relative distances between the Fab and Fc fragments, it was not possible to detect differences in the scattering curves of the two isotypes. The combination of neutron-scattering data on the purified isotypes and an automated curve-fitting procedure was then able to show for the first time that it is possible to rationalize IgG scattering curves directly from Fab and Fc crystal structures.

The application of automated curve-fitting procedures starting from atomic co-ordinates has considerable potential for improving the interpretation of X-ray and neutron-scattering studies of multidomain proteins. All sterically allowed arrangements of domains can be explored to give a limited family of structures allowed by the scattering curve. The method is able to estimate the experimental precision to which a structure can be determined by scattering, since the models that give good curve fits can be used to determine error ranges. The utility of this method is that it should be able to exclude well over 90% of possible structures from further consideration. As solution scattering does not lead to a unique structure determination, it can only show structures that are consistent with the experimental curves. It is possible that some of the fitted conformations may

Figure 6 Alignment of heavy-chain sequences of ruminant and porcine IgG [7,10] with those of crystallographically determined Fab and Fc fragments

Sequences for porcine IgG1, IgG2a, IgG2b, IgG3 and IgG4 are based on Genbank sequence accession codes U03778, U03779, U03780, U03781 and U03782. The Fab and Fc crystal structures are indicated by their Brookhaven database codes. For all the sequences, only the regions of the C_H1 and C_H2 domains flanking the hinge region are shown. Residues are numbered if they correspond to the β -sheet structure of the Fab and Fc fragments, and are lettered if otherwise in the 'genetic' hinge or 'lower' hinge regions. Residue conservation between the ruminant sequences and the crystal structure sequences is indicated with asterisks when this is over 90%. The Glu³¹⁸-Lys³²⁰-Lys³²² motif in the C_H2 domain that interacts with C1q of complement occurs at β -strand F (not shown). Secondary-structure analyses using the program DSSP are shown in the same alignment as their sequences. The seven β -strands A to G of the immunoglobulin fold [42] correspond to strands fx1, fx2, fy1, fx3, fy4, fy2 and fy3 in that order [44]. Secondary-structure elements when detected by DSSP are abbreviated: E, extended β -strand (in bold); H, α -helix; B, bend; S, disordered; T, turn; G, 3_10 -helix.

not exist. In application to IgG, the dynamic aspects of IgG structure in terms of Fab and Fc flexibility about the hinge should be noted [48]. Microscopy and fluorescence studies have characterized the existence of variable hinge angles or rotational motions in IgG, but these have not been considered in the present study. It should also be noted that, although a planar IgG model was satisfactorily generated from the present curve-fitting procedure, asymmetric non-planar IgG structures have not been considered. The effects of dynamic or non-planar structures are, however, expected to be minimal on the outcome of the present study, on the assumption that IgG occupies a single predominant population in solution.

As described previously [7], the hinge sequence of bovine IgG2 requires a mainly extended conformation in order to link the Fab and Fc fragments. This results in a model in which the spatial distance between the α -carbon of the Cys residue at the C-terminus of the C_H1 domain and the Pro residue at the N-terminus of the C_H2 domain lies between 3.0 and 3.2 nm. In contrast, the hinge sequence of bovine IgG1 can be given a range of conformations in which the above spatial distance lies between 3.6 and 5.3 nm. The lower values result from shortening the hinge sequence as much as possible with two reverse turns in the polyproline segment. The scattering results described in this paper are best fitted to a compact IgG1 molecule with the coiled hinge conformation. Presumably a molecule with an extended hinge conformation would be at a biological disadvantage, probably because of the remoteness of the Fab fragments from the Fc fragment. When considering possible relationships between hinge sequences and effector functions, it should be borne in mind that a long hinge sequence does not necessarily result in a large spatial distance between the Fab and Fc fragments. Although modelling suggested that the spatial distance between the C-termini and N-termini of the Fab and Fc fragments may be less in IgG2 than in IgG1, the postulated difference of about 0.6 nm is not detectable by the present technique.

In earlier studies of bovine [7] and ovine [10] IgG, we have proposed that the combined effect of hinge shortening (deletion) and steric restriction at the C_H1 /hinge/ C_H2 interface is the probable basis for the functional differences in mammary transport between the ruminant isotypes. Both bovine and ovine IgG1 and IgG2 are able to fix complement with similar effectiveness [49,50], and this is not in conflict with the concept of different hinge spacing between them, as the complement-binding sequence motif Glu³¹⁸-Lys³²⁰-Lys³²² [51] is present and accessible in the C_H2 domain of both isotypes (Figure 5). In contrast, the conserved Fc γ RI-type receptor motif LLGGP is modified to LPGGP in IgG1 and is deleted in IgG2 (Figure 5). The implications of these changes for Fc receptor function are discussed by Symons and Clarkson [52]. The ruminant mammary receptor for IgG1 and its motif on the Fc fragment are not yet identified, but it remains likely that the deletions and sterically shortened hinge of IgG2 are the elements that exclude this isotype from the transport process.

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