The Binding of Lanthanides to Non-Immune Rabbit Immunoglobulin G and Its Fragments

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The binding of Gd(III) to rabbit IgG (immunoglobulin G) and the Fab (*N*-terminal half of heavy and light chain), (Fab')₂ (*N*-terminal half of heavy and light chains joined by inter-chain disulphide bond), Fc (*C*-terminal half of heavy-chain dimer) and pFc' (*C*-terminal quarter of heavy-chain dimer) fragments was demonstrated by measurements of the enhancement of the solvent-water proton relaxation rates in the appropriate Gd(III) solutions. At pH 5.5 there are six specific Gd(III)-binding sites on the IgG. These six sites can be divided into two classes; two very 'tight' sites on the Fc fragment (K_d approx. $5\ \mu\text{M}$) and two weaker sites on each Fab region (K_d approx. $140\ \mu\text{M}$). Ca(II) does not apparently compete for these metal-binding sites. The metal-binding parameters for IgG can be explained as the sum of the metal binding to the isolated Fab and Fc fragments, suggesting that there is no apparent interaction between the Fab and Fc regions in the IgG molecule. The binding of Gd(III) to Fab and Fc fragments was also monitored by measuring changes in the electron-spin-resonance spectrum of Gd(III) in the presence of each fragment and also by monitoring the effects of Gd(III) on the protein fluorescence at 340 nm (excitation 295 nm). The fluorescence of Tb(III) solutions of 545 nm (excitation 295 nm) is enhanced slightly on addition of Fab or Fc.

As part of our programme to understand the mechanism by which the sequence of reactions of the proteins of the complement system is triggered after the formation of the immune complex, we have been looking at a series of spectroscopic probes. The chance finding that the mouse myeloma protein MOPC 315 had one specific lanthanide-binding site in the Fv* region (Dwek *et al.*, 1975a) has prompted us to search for such metal-binding sites on antibodies. The attraction of using the lanthanide ions as probes is that they comprise a series of fifteen elements. All the cations M(III) have much the same chemical properties, so that they are expected to bind to the same site(s), though the subtle graded differences between the members of the series allow detailed inspection of some properties of the site. The series of lanthanide cations carry into the site, by single-atom for single-atom substitution, a wide range of spectroscopic probes. Thus the lanthanides can be used as n.m.r. (nuclear

magnetic resonance), fluorescence and e.s.r. (electron spin resonance) probes, and further, they are useful as heavy atoms for X-ray-crystallographic studies.

The primary aim of the work reported here, on non-immune rabbit IgG and various associated fragments, is therefore to screen the antibody for lanthanide-binding sites which may be useful in studying antibody-antigen complexes, and also the subsequent interaction with the complement system of proteins. In the classical pathway, the activation of this sequence involves the Fc region of the antibody molecule and thus it is extremely important to have probes in this region.

Many of the studies reported here are concerned with the binding of Gd(III) to IgG, and its various fragments, by monitoring the changes in the solvent-water proton relaxation rates. In general, the binding of paramagnetic ions such as Gd(III) to proteins often results in significant enhancements of these relaxation rates (Dwek, 1973). The observed enhancement depends on the amount of metal ion bound to the protein and thus, like any spectroscopic technique, the dissociation constant for the metal ion-protein complex can be determined. However, there is an additional advantage of this technique over others in that the enhancement of the complex is related to intrinsic structural parameters of the binding site. Any changes occurring at the

* Abbreviations: Fv fragment, variable region of heavy and light chain, denoted V_{HL} in Scheme 1. IgG, immunoglobulin G; Fab fragment, *N*-terminal half of heavy chain and light chain; (Fab')₂, *N*-terminal half of heavy chain and whole of light chain joined by inter-heavy-chain disulphide bond; Fc, *C*-terminal half of heavy-chain dimer; pFc', *C*-terminal quarter of heavy-chain dimer. These fragments are shown in Scheme 1 (V_{HL}, C_{H1CL}, C_{H2}, C_{H3} are the different domains).

Scheme 1. Structure of IgG

Proteolytic fragments of rabbit IgG. Papain hydrolysis cleaves IgG into two Fab fragments and one Fc fragment. Pepsin cleavage gives one $(\text{Fab}')_2$ fragment, including the inter-heavy-chain disulphide link, and several peptides representing splits in the Fc region, among which was pFc' . The different domains are V_{HL} , CH_1CL , CH_2 and CH_3 and are shown by circled regions.

metal site, for example, through a conformational change will cause this enhancement to alter (Dwek, 1973). Such probes are thus likely to prove useful eventually in answering the controversial question as to whether the binding of antigen to antibody causes conformational changes in the Fc region (Muller-Eberhard, 1971).

Materials and Methods

Isolation of rabbit IgG and fragments

IgG was prepared from pooled non-immune serum (Wilkinson, 1969). The Fab and Fc fragments (see Scheme 1) were prepared by the digestion of IgG (15mg/ml) with papain (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) in 100mM-Tris-HCl, pH7.2, in the presence of 10mM-cysteine and 2mM-EDTA at an enzyme/substrate ratio of 1:50 (w/w), for 20min at 37°C. Iodoacetamide was

added to 20mM and the digest immediately fractionated on a column (55cm $\times$ 5cm) of Sephadex G-100 equilibrated with 60mM-sodium acetate, pH5.6, to separate the fragments from undigested IgG. The fragments were then chromatographed on a column (30cm $\times$ 3cm) of CM-cellulose (CM-32; Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) in the same buffer. The Fab fragment was not adsorbed and the Fc fragment was eluted with a salt gradient to 500mM-sodium acetate, pH5.6. Both fragments were exhaustively dialysed against water and freeze-dried. The purity was checked by double diffusion in agar (Ouchterlony, 1953) with specific antisera and also by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. Digestion of IgG with activated papain in the absence of cysteine for 4h at 37°C, followed by fractionation on columns of Sephadex G-100 and CM-cellulose as above, yielded identical Fab fragments. The Fc fragments, however, differed in their content of disulphide-linked dimer. Fc fragment prepared in the presence of 10mM-cysteine was a 50:50 mixture of disulphide-bond linked dimer and non-covalently bound dimer, whereas Fc fragment prepared in the absence of cysteine was shown by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate to be 85% in the form of a covalently linked dimer.

$(\text{Fab}')_2$ and pFc' fragments (see Scheme 1) were prepared by digestion of IgG (20mg/ml) in 100mM-sodium acetate, pH4.5, with pepsin (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) at an enzyme/substrate ratio of 1:50 (w/w) for 16h at 37°C. The digest was fractionated on a column (90cm $\times$ 5cm) of Sephadex G-200 in 100mM-sodium phosphate buffer, pH6.8. Only the five fractions around the peaks of the $(\text{Fab}')_2$ and pFc' fragments were taken for further study. Both fragment fractions were concentrated to 15–20mg/ml by ultrafiltration and dialysed extensively against 0.15M-NaCl, pH5.4. The $(\text{Fab}')_2$ fragment contained less than 1% Fc fragment, as judged by double diffusion in agar with anti-Fc antisera, and the pFc' fragment gave the expected amino acid composition (Prahl, 1967).

Preparation of metal solutions

Solutions of Gd(III) were prepared by roasting Gd_2O_3 (99% pure; Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) in air at 600°C for 12h and dissolving in 10% (v/v) HCl, to 10mM. For titrations the stock solution was diluted with water and adjusted to pH5.5 by addition of NaOH. To minimize the effect of Gd(III) binding to glassware (Jones *et al.*, 1974), all n.m.r. tubes were silicone-treated before use (Repelcote; Hopkin and Williams Ltd., Romford, Essex, U.K.; laboratory reagent; 2% solution of dimethyl dichlorosilane in carbon tetrachloride) and where possible plastic pipettes

were used. All experiments were performed in 0.15M-NaCl or -KCl (AR reagents; Fisons, Loughborough, Leics., U.K.) to simulate physiological conditions. For competition experiments, suitable dilutions in water from a stock 0.3M-CaCl₂ solution (Fisons AR reagent) were used.

Proton relaxation and e.s.r. (electron spin resonance) measurements

Proton spin lattice relaxation times were measured at 20 MHz on a spin echo machine constructed in this laboratory. The ambient temperature was $19 \pm 1^\circ\text{C}$. E.s.r. spectra were recorded on a J.E.O.L. JES-PE spectrometer at X band.

Fluorescence measurements

Fluorescence measurements were made on a Perkin-Elmer Hitachi MPF 2A spectrofluorimeter equipped with a thermostatically controlled cell compartment at 25°C .

Ultracentrifugation experiments

Sedimentation-velocity experiments were performed on a Beckman model E analytical ultracentrifuge. Boundaries were photographed by using schlieren optics. The runs were carried out at $19 \pm 1^\circ\text{C}$ and 56100 rev./min. Approximate sedimentation coefficients were obtained from comparator measurements of a series of photographs taken at 8 min intervals.

Theoretical treatment of proton relaxation results

The introduction of a protein to which a paramagnetic ion like Gd(III) binds can lead to an increase in the water proton relaxation rates (Dwek, 1973). For a single class of equivalent binding sites, the paramagnetic contribution to the water proton spin lattice relaxation ($1/T_{1P}$) of a metal ion solution containing an arbitrary amount of protein may be written as (Dwek, 1973):

$$\frac{1}{T_{1P}} = \frac{(1 - X_B)}{T_{1F}} + \frac{X_B}{T_{1B}} \quad (1)$$

where $1/T_{1F}$ is the relaxation rate of the water protons in a solution containing only the metal ions, and $1/T_{1B}$ is the relaxation rate in a solution containing the same amount of metal ion bound to protein. $X_B \equiv [M_B]/[M_T]$ is the fraction of metal ion bound to the protein, and is an explicit function of the number of equivalent binding sites, n , and the intrinsic dissociation constant, K_d , of the metal ion from each equivalent protein-binding site. For n

equivalent binding sites the concentration of bound metal, $[M_B]$, is given by the expression:

$$[M_B] = \frac{n[P_T][M_F]/K_d}{\left(1 + \frac{[M_F]}{K_d}\right)} \quad (2)$$

where $[P_T]$ is the total protein and $[M_F]$ is free metal ion concentration. Thus X_B is given by the expression:

$$X_B \equiv \frac{[M_B]}{[M_T]} = \frac{n[P_T]}{K_d \left(1 + \frac{[M_F]}{K_d}\right) + n[P_T]} \quad (3)$$

where $[M_T]$ is total metal ion concentration.

Two different types of experiment have been performed; one in which the water proton spin lattice relaxation rate is followed at constant metal concentration and various protein concentrations (P-titration), and the other at constant protein concentration and various metal concentrations (M-titration). P-titrations are carried out over a range of protein concentration such that the limiting protein concentrations are in excess over that of the metal. This means that P-titrations will not show up any substantially weaker binding processes occurring at secondary sites. On the other hand, M-titrations, in saturating the protein with metal, will show weaker binding sites as well.

P-titrations are analysed by using eqns. (1)–(3). $1/T_{1F}$ is obtained from the value of the relaxation rate in the absence of protein and is thus directly measurable. The only unknown parameters are then K_d , n and $1/T_{1B}$. Since $[M_B] = [M_T] - [M_F]$, eqn. (2) may be solved by the standard methods to give the free metal ion concentration, $[M_F]$, in terms of the total metal ion concentration, $[M_T]$, the intrinsic dissociation constant, K_d , and the number of binding sites, n . Inserting this solution of eqn. (2) into eqn. (3) thus gives X_B as a function of two experimentally controlled parameters, $[P_T]$ and $[M_T]$, and two adjustable parameters, K_d and n . Eqn. (3) can thus be inserted into eqn. (1) to give a theoretical expression for the experimentally observed relaxation rate ($1/T_{1P}$) in terms of three adjustable parameters, K_d , n and $1/T_{1B}$.

P-titrations may be analysed by using eqn. (1) directly. However, in the M-titrations the value of $1/T_{1F}$ and $1/T_{1B}$ continually change throughout the titration as the total metal ion concentration is varied. It is convenient to define

$$\frac{1}{T_{1F'}} \equiv \frac{1}{[M]} \cdot \frac{1}{T_{1F}}$$

where $1/T_{1F'}$ is the relaxation rate in a solution containing a concentration, $[M]$, of free metal ion. Similarly

$$\frac{1}{T_{1B'}} \equiv \frac{1}{[M]} \cdot \frac{1}{T_{1B}}$$

where $1/T_{1B}$ is the relaxation rate in a solution containing a concentration $[M]$ of bound metal ion. Eqn. (1) may thus be rewritten:

$$\frac{1}{T_{1P}} - \frac{1}{T_{1F}} = \left(\frac{1}{T_{1B'}} - \frac{1}{T_{1F'}} \right) [M]_B \quad (4)$$

where $(1/T_{1P} - 1/T_{1F})$ is the difference between the relaxation rate of the metal ion solution in the presence and the absence of the protein. Eqn. (2) can be solved by standard methods to give $[M]_B$ in terms of n and K_d . Inserting this solution to eqn. (2) into eqn. (4) thus gives $(1/T_{1P} - 1/T_{1F})$ in terms of two experimentally controlled parameters, $[P_T]$ and $[M_T]$, and three unknown parameters, K_d , n and $1/T_{1B'}$. Obviously $1/T_{1F}$ and $1/T_{1B}$ in eqn. (1) are trivially related to $1/T_{1F'}$ and $1/T_{1B'}$ in eqn. (4).

In general, it is difficult to fit unequivocally either the M-titration or the P-titration to unique values of the three parameters n , K_d and $1/T_{1B}$ ($1/T_{1B'}$). However, the data from both an M-titration and a P-titration may be simultaneously fitted to give unique values of the three parameters. The only problem involves the presence of weak binding sites which may appear in the M-titration. We should note that to obtain $1/T_{1P}$ from the observed relaxation rate of the solution ($1/T_{1(obs.)}$) the relaxation rates of the corresponding solutions in the absence of the paramagnetic metal ion ($1/T_{10}$) must also be measured. By using an asterisk to denote the presence of the protein, $1/T_{1P}$ and $1/T_{1F}$ can be obtained from the following equations:

$$\frac{1}{T_{1P}} = \frac{1}{T_{1(obs.)^*}} - \frac{1}{T_{10^*}}$$

$$\frac{1}{T_{1F}} = \frac{1}{T_{1(obs.)}} - \frac{1}{T_{10}}$$

Both types of titration can also be treated by the method of Scatchard (1949) by using the equations:

$$\frac{\bar{\mu}}{[M_F]} = \frac{1}{K_d} (n - \bar{\mu}) \quad (5)$$

and

$$[M_B] = \frac{(1/T_{1P} - 1/T_{1F})}{(1/T_{1B} - 1/T_{1F})} [M_T] \quad (6)$$

where $\bar{\mu}$ is the average number of metal ions bound/molecule or protein. We note that the calculation of $[M_B]$ involves an initial estimation of $1/T_{1B}$ obtained from the plateau region of P-titrations. The Scatchard plots were then calculated by smoothing the experimental points from P- and M-titrations to the best continuous curve through all the data. The values of n and K_d so obtained were then used as initial estimates for the theoretical curve-fitting procedures described above from which accurate estimates of the binding parameters can be made.

Since a P-titration on any one fragment does not include any contribution from secondary weaker metal-ion binding sites, any weak sites which show up in the M-titration data cannot be treated quantitatively unless a value of $1/T_{1B}$ appropriate to these sites can be estimated. For example, IgG shows two main types of binding site: two sites similar to Fc, and a number of other sites, which we have treated for the purposes of analysis, with the value of $1/T_{1B}$ of the two metal-binding sites found for the isolated Fab fragment. Since there may be strong interaction between the domains in the IgG molecule there is presumably no reason why $1/T_{1B}$ for a distinct site will remain constant in the different fragments, and so this procedure must be treated with caution. Nevertheless such a treatment for the weaker sites of IgG fits a value of $n = 4$ and $K_d = 150 \mu M$, with some even weaker sites similar to the weak sites found on the Fab fragment M-titrations. This agreement may suggest that this procedure is valid in this particular case.

It is often more convenient, particularly when comparing results from P-titrations at different amounts of paramagnetic ions, to refer the measured rates to those in the corresponding solutions of the paramagnetic ion in which the protein is absent. Thus it is usual to define the enhancement (ϵ_b) as:

$$\epsilon_b \equiv \frac{1/T_{1B}}{1/T_{1F}} \quad (7)$$

Results and Discussion

Proton relaxation studies

Fab. Water proton spin lattice relaxation rates were measured at a constant Gd(III) concentration and various protein concentrations (P-titration; Fig. 1) and at a constant protein concentration and various metal concentrations (M-titration; Fig. 2). Taking a value of ϵ_b of 2.7 from the plateau region of the P-titration, a Scatchard plot can be calculated from M-titration data and is presented in Fig. 3. The plot shows two 'tight' Gd(III)-binding sites, and also a number of weaker sites. Theoretical curves were then calculated over a series of six experiments and the values of n , K_d and ϵ_b obtained are shown in Table 1. The results are consistent only with a stoichiometry of two binding sites/Fab molecule, though all the M-titrations also display a number of weaker sites, as shown by the deviation of the theoretical curve from the experimental points at high ($>800 \mu M$) Gd(III) concentration. For reasons outlined in the theoretical section, these weaker sites cannot in this case be treated quantitatively. The value of K_d calculated for the two 'tight' sites assumes that they are equivalent and, from the fit of the experimental points to the theoretical curves this would

Fig. 1. Titration of Gd(III) with Fab fragments by following the paramagnetic contribution to the water proton spin lattice relaxation rate (P-titration)

The continuous curve is calculated theoretically by assuming $n = 2$, $K_d = 150 \mu\text{M}$, $\epsilon_b = 2.7$; Gd(III) concentration, $115 \mu\text{M}$; other conditions: NaCl concentration, 0.15M ; pH 5.5 ; temperature, $19 \pm 1^\circ\text{C}$.

Fig. 2. Titration of Fab fragments with Gd(III) by following the difference between the paramagnetic contribution to the water proton spin lattice relaxation rate in the presence and absence of Fab fragments

The continuous curve is calculated theoretically for the following binding parameters: $n = 2$; $K_d = 200 \mu\text{M}$; $\epsilon_b = 2.7$; Fab fragment concentration, $210 \mu\text{M}$. Other conditions were as for Fig. 1.

appear a reasonable assumption, though some non-equivalence cannot be ruled out.

Water proton spin lattice relaxation rates were measured with Mn(II) instead of Gd(III) over a similar concentration range. No change in the relaxation rate was observed as a function of Fab concentration, suggesting that the binding of Mn(II) to this fragment is very much weaker than the binding of Gd(III).

Fig. 3. Scatchard (1949) plot for Gd(III) binding to Fab fragments

The points are derived from an M-titration where the Fab fragment concentration was $210 \mu\text{M}$. ϵ_b was taken as 2.7 . Other conditions were as for Fig. 1.

Table 1. Summary of parameters obtained from proton-relaxation studies of Gd(III) binding to IgG and its fragments

The values of K_d are mean values. The errors represent the maximal variations over all the experiments.

Fragments	n	K_d (μM)	Range of ϵ_b values	No. of expts.
Fab	2	140 ± 60	2.7–3.2	6
(Fab') ₂	4	100 ± 50	(0.4, 2.7, 3.0)*	9
Fc	2	6 ± 3	6.3–7.0	6
pFc'	1	4	8.2	1
IgG	2	6 ± 2	9.5	3
	4	200 ± 50	2.7–3.2	

* Values of 0.4 and 3.0 obtained for freeze-dried preparations

pH-dependence of relaxation rates. Lanthanide solutions are stable only up to pH 7, because the formation of hydroxide complexes, with resulting precipitation. Even at pH 6, extensive hydroxide complex-formation occurs. It is to be expected that lanthanides will bind to carboxyl groups on antibody and antibody fragments. Such groups generally have pK_a values in the range pH 4–6. Hence, to optimize the conditions for observation of metal binding to antibody fragments, measurements were obtained over the pH range 3.5–6.5. The results are shown in Fig. 4. The half-way point of the curve is at pH 4.6, which is in the range expected for a

Fig. 4. pH-dependence of the paramagnetic contribution to the water proton spin lattice relaxation rates

Gd(III) concentration was $143\mu\text{M}$. Fab fragment concentration was $500\mu\text{M}$. Other conditions were as for Fig. 1. The relaxation rate of the free aqueous Gd(III) solution alone at pH 3.0 is shown for reference.

carboxyl group(s). It must be emphasized that this is not a true titration curve, as preliminary experiments indicated that both the enhancement and the apparent binding constant are affected by pH. However, one can see that the pH chosen for further titration (pH 5.5) is in the plateau region of the curve, where the maximum effects are observed.

(Fab')₂. A similar series of experiments to those on Fab fragment has also been carried out on the (Fab')₂ fragment. A representative Scatchard plot, calculated from an M-titration, with an ϵ_b of 2.7 from P-titration data, shows (Fig. 5) four 'tight' binding sites and some other weaker sites. A summary of the results of theoretical curve fitting to nine experiments on three different preparations of this fragment is shown in Table 1. A variation in ϵ_b was found for the three preparations. It was thought that this may be due to slight changes in protein conformation occurring during the freeze-drying procedure. Thus the third preparation (see Fig. 5), was not freeze-dried. That the differences in ϵ_b did not arise from changes in aggregation of the fragment was established by ultracentrifugation experiments (see below). However, in all nine experiments, the theoretical curve-fitting procedures gave a stoichiometry of four binding sites per (Fab')₂ dimer and a K_d for each site of approx. $100\mu\text{M}$. Gd(III) binding to (Fab')₂ can thus be explained as

Fig. 5. Scatchard plot for Gd(III) binding to (Fab')₂ fragment

The points are derived from an M-titration where (Fab')₂ fragment concentration was $100\mu\text{M}$. ϵ_b was taken as 2.9. Other conditions were as for Fig. 1.

Fig. 6. Scatchard plot for binding of Gd(III) to Fc fragments

The points are derived from an M-titration where Fc fragment concentration was $54\mu\text{M}$. ϵ_b was taken as 6.4. Other conditions were as for Fig. 1.

the sum of the binding to monomeric Fab fragments. There is therefore no evidence for interactions between Fab fragments in the (Fab')₂ dimer.

Fc. A similar series of experiments to those with Fab fragments was also carried out on Fc fragments. The Scatchard plot, calculated from M-titration data, with an ϵ_b of 6.4 from P-titrations, shows (Fig. 6) two very 'tight' binding sites with a K_d of approx. $8\mu\text{M}$ and a number of weaker sites. Curve fitting to all six experiments confirms this result (Table 1). Particularly noticeable are the much higher value of ϵ_b and much tighter binding than seen for the Fab or (Fab')₂ fragments.

The Fc fragment will only dissolve readily at pH 4, and therefore solutions of Fc fragments are usually

Fig. 7. Scatchard plots for binding of Gd(III) to IgG from (a) P-titration and (b) M-titration data

For (a) Gd(III) concentration was $143 \mu\text{M}$. For (b) the IgG concentration was $30 \mu\text{M}$. For both (a) and (b) ϵ_b was taken as 9.5. Other conditions were as for Fig. 1.

made up in 10mm-acetic acid. It was possible that the acetic acid was itself binding to Gd(III) and producing spurious contributions to the enhancement. Experiments were thus performed in which the Fc fragment was dissolved by adjustment of the pH with HCl and NaOH. The results for n , K_d and ϵ_b were within the limits of the previous experiments done in 10mm-acetic acid. We conclude that acetate has no effect on the binding parameters.

Further, it was possible that the reduction and alkylation step in the preparation of Fc fragments (which results in alkylation of 50% of the S-S bonds in the preparation) was slightly altering the Gd(III)-binding parameters, in particular ϵ_b . Also there was

a possibility that the iodoacetamide solution contained a significant proportion of iodoacetate, in which case carboxymethyl groups might be added to the protein to present a potential binding site for Gd(III). Experiments with an Fc preparation that had not been treated with iodoacetamide, and which was 80% S-S-bonded, gave results within the experimental limits for iodoacetamide-treated preparations. (Additionally, experiments with an Fab preparation that had not been treated with iodoacetamide gave similar results to previous experiments with this fragment.)

In view of the requirement for Ca(II) in the interaction of complement with the Fc region, a Ca(II)-competition experiment was performed by addition of a series of concentrations of Ca(II) to a Gd(III)/Fc solution up to a 400-fold excess of Ca(II) over Gd(III). Over the whole range studied, the relaxation rate was unchanged by the addition of Ca(II) except for that accounted for by dilution. Thus Ca(II) does not appear to compete for the Gd(III)-binding site on Fc fragments.

pFc'. A preliminary observation of the binding of this fragment by P-titration is consistent with a single 'tight' binding site with a K_d of approx. $4 \mu\text{M}$.

IgG. By using the value of ϵ_b (9.5) derived from the P-titration, Scatchard plots were calculated from P- and M-titration data; these are shown in Figs. 7(a) and 7(b). The Scatchard plot from the P-titration, shows two 'tight' sites (K_d 4–8 μM). The Scatchard plot from the M-titration shows these two sites and a further three to five weaker sites. Since the P-titration contains contributions only from the 'tight' sites, the ϵ_b from these titrations cannot be used in a quantitative analysis of the weaker binding processes. Hence one cannot estimate K_d for these weaker sites from the M-titration Scatchard plot, which is constructed by using this ϵ_b value.

The best-fit parameters for the M-titration data were derived by using the computational procedure described above. The binding only to the 'tight' sites is estimated over a range of total metal concentration $[\text{M}_T]$, and then by using the remaining free metal concentration at each $[\text{M}_T]$ value, the binding to the weaker sites is initially estimated by using ϵ_b and K_d values that were found for Fab fragments. The ϵ_b and K_d values are then further refined. The parameters so obtained for the weak sites are $\epsilon_b = 3.0$, $K_d = 150 \mu\text{M}$ and $n = 4$ (see Fig. 8). Gd(III) binding to IgG can then be explained as the sum of the binding to isolated Fab and Fc fragments. There is then no evidence for interaction between the Fab and Fc regions in the intact IgG molecule.

A hypothesis consistent with all the results on metal binding is that each antibody molecule contains six 'tight' Gd(III)-binding sites and that these are arranged as one site on each domain of the molecule (Scheme 1). These 'tight' sites appear to divide into

Fig. 8. Titration of IgG with Gd(III) by following the difference between paramagnetic contribution to the water proton spin lattice relaxation rates in the presence and absence of IgG

●, Experimental curve; ○, theoretical curve calculated for $n = 2$, $K_d = 4 \mu\text{M}$, $\epsilon_b = 9.5$; △, theoretical curve calculated for $n = 4$, $K_d = 200 \mu\text{M}$, $\epsilon_b = 2.7$; ▲, sum values from ○ and △ data. IgG concentration was $30 \mu\text{M}$. Other conditions were as for Fig. 1.

two classes. On each of the two domains of the Fc region there is one 'tight' binding site (K_d approx. $5 \mu\text{M}$). This is shown by the results for fragments Fc ($n = 2$) and pFc' ($n = 1$). On each Fab arm of the molecule, there appear to be two sites of K_d approx. $140 \mu\text{M}$. This is shown by the results for both fragments Fab ($n = 2$) and (Fab')₂ ($n = 4$). Though the experiments here do not rigorously prove that each of these Fab sites is on a separate domain, previous experiments on the Fv fragment (region denoted V_{HL} in Scheme 1) from the mouse myeloma protein MOPC 315, which show one lanthanide-binding site (Dwek *et al.*, 1975b), suggest that this is the case, though some caution is obviously needed in comparing results from different species.

Temperature-dependence of relaxation rates of IgG, and Fc fragments and Gd(III)

The water proton spin lattice relaxation rate was measured as a function of temperature over the range 273–350°K for IgG, Fab and Fc solutions containing Gd(III). Under the experimental conditions used, most of the Gd(III) is bound. The results in Fig. 9 show that the relaxation processes for the water protons in the Gd(III)–IgG complex are quite different from those in the Gd(III)–Fab complex. Comparisons of the results for Gd(III)–Fc and Gd(III)–IgG complexes, however, show that in these two cases

Fig. 9. Temperature variation of the water proton spin lattice relaxation rate for IgG, Fc and Fab fragments in the presence of Gd(III) and for Gd(III) alone

●, $143 \mu\text{M}$ -Gd(III). ■, $400 \mu\text{M}$ -Fab fragment + $143 \mu\text{M}$ -Gd(III). □, $190 \mu\text{M}$ -Fc fragment + $143 \mu\text{M}$ -Gd(III). ○, $200 \mu\text{M}$ -IgG + $143 \mu\text{M}$ -Gd(III).

the relaxation processes are qualitatively similar. However, frequency and temperature-dependence studies should clarify the details of the relaxation mechanism, and also the difference between the relaxation mechanism for Gd(III) bound to Fab and Fc fragments, respectively. It should be noted that the data in Fig. 9 are not corrected for the temperature-dependence of the buffer solution. The correction would not, however, affect the above qualitative conclusions.

E.s.r. studies

Fab. The line height of the gadolinium e.s.r. signal was observed, as a function of gadolinium concentration in the presence and absence of Fab fragments. The results are shown in Fig. 10. In the presence of Fab fragments there is a break at a Gd(III)/Fab concentration ratio of approx. 2:1. This is consistent with the value of 2:1 found in the proton relaxation studies described above. The insensitivity of this technique, caused by the broad line width of the Gd(III) e.s.r. signal, requires the concentrations of Gd(III) and protein to be higher than those used in the proton-relaxation studies. Further, it is difficult to obtain an accurate value of K_d from this technique because the deviations from linearity in the titration curve are of the same order as the experimental error.

It may be noted from Fig. 10 that the slope of the line above the break point is greater than that for

Fig. 10. Titration of Gd(III) fragment with Fab by following the line intensity of the Gd(III) e.s.r. signal

The lines represent the line intensity over a range of Gd(III) concentrations, in the presence of 500 μM-Fab fragment (□) or 10mM-acetate (●) or aq. Gd(III) alone (○). Other conditions were as for Fig. 1.

solutions of aqueous gadolinium. This may result from the presence of acetate bound to the Fab fragments, since acetate buffer is used in the preparation of Fab fragments. It can be shown that the binding of acetates of Gd(III) causes an increase in the line height of the e.s.r. signal (see Fig. 10).

Fc. The result of an experiment observing the line height of the Gd(III) e.s.r. signal with increasing Gd(III) concentration in the presence of constant Fc fragment concentration, is shown in Fig. 11. There is a definite break in the plot at a Gd(III)/Fc concentration ratio of approx. 2:1. This is consistent with the value of $n = 2$ found in the proton-relaxation studies. In the studies of Fc fragments, 10mM-acetate was added as described in the proton-relaxation section. There is an advantage in doing this for the e.s.r. studies, since acetate increases the line height of the Gd(III) e.s.r. signal and this facilitates the observation of the break point seen in Fig. 11. This is because the linewidth of the bound Gd(III) e.s.r. signal in the Fc complex is very different from the value when Gd(III) is bound to acetate. The similarity in line widths between that for free aqueous Gd(III) and for the Gd(III)-Fc complex would make this experiment impractical in the absence of acetate. For reference, a plot of line height as a function of Gd(III) concentration in the presence of 10mM-acetate alone is shown in Fig. 11. Control experiments were carried out in which the variations of both line height and linewidth of the Gd(III) e.s.r. signal were observed as

Fig. 11. Titration of Gd(III) with Fc fragments by following the line intensity of the Gd(III) e.s.r. signal

The lines represent the line intensity over a range of Gd(III) concentrations in the presence of 10mM-acetate and 160 μM-Fc fragment (○) and in the absence of Fc fragment (●). Other conditions were as for Fig. 1.

Table 2. Summary of sedimentation coefficients obtained for IgG and its fragments in the presence and absence of saturating amounts of Gd(III)

Fragments	$s_{20,w}$ (S)	
	Without Gd(III)	With Gd(III)
Fab	3.6	3.8
(Fab') ₂	4.9	4.5
Fc	3.8	4.2
IgG	6.9	7.3
pFc'	2.6	2.4

a function of acetate concentrations over a range 0–10mM-acetate. These gave an effective K_d value for the Gd(III)-acetate complex of approx. 2mM. Hence under the conditions used in these experiments nearly all the Gd(III) not bound to Fc fragments will effectively be bound to acetate.

Fluorescence studies

The effect of Gd(III) on the protein fluorescence of Fab and Fc fragments at 340nm (excitation 295nm) was monitored at pH 5.5 in 0.15M-NaCl. An overall increase in fluorescence intensity of approx. 10% was found in these experiments. Similar concentrations of Gd(III) had no effect ($\pm 1\%$) on the fluorescence intensity of a tryptophan blank under these conditions. The changes observed in these experiments, however, are too small to estimate reliable quantitative values of K_d .

Preliminary experiments also indicate an enhancement of terbium fluorescence at 545nm (excitation 295nm) on addition of Fab or Fc fragments. An

enhancement (pH 5.5, 0.15M-NaCl) of approx. 100 is calculated after correction to an appropriate protein blank.

Ultracentrifugation studies

Sedimentation-velocity experiments were carried out on whole IgG and on each of the antibody fragments in both the presence and the absence of a saturating amount of Gd(III). The results (see Table 2) showed that under the experimental conditions used in this work (P- or M-titrations) there was no significant aggregation of IgG or its fragments and further that any Gd(III)-induced effects on the $s_{20,w}$ values are small and likely to be unimportant.

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