PROTON RELAXATION ENHANCEMENT TO PROBE THE EFFECTS OF ANTIGEN BINDING ON PIG ANTIBODY STRUCTURE

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1. Introduction

There is still an unresolved problem as to whether occupation of antibody binding sites by antigen induces conformational changes in distant parts of the antibody molecule. The problem is, amongst others, pertinent for the elucidation of the mechanism of complement activation (reviewed in [1]). A useful approach to the study of the problem is to introduce probes in specific regions of the antibody molecule and look for changes detected by the probe on antigen binding. The versatility of paramagnetic probes in biological systems is now well-documented [2], an experimentally easy technique being to monitor the effect of the paramagnetic probe on solvent water proton relaxation (proton relaxation enhancement (PRE)) under various conditions. We have characterized the binding of the paramagnetic ion Gd(III) to rabbit IgG using the PRE technique [3]. Further, we have carried out detailed molecular motion studies on the IgG/Gd(III) complex using PRE [4,5] which revealed an unusual degree of mobility in the Fc region of the antibody molecule.

In order to use PRE to probe possible changes in antibody conformation occurring on antigen binding a number of conditions should be fulfilled:

(i) A specific antibody must be used;
(ii) The antigen chosen should not bind the paramagnetic ion to any significant extent;
(iii) It would be desirable to work at antigen to antibody ratios where no precipitation occurs.

Here the paramagnetic ion chosen was Gd(III), the antibody was either an early (precipitating) or late (non-precipitating) pig anti-Dnp antibody and the antigens were Dnp_{14}– or mono-Dnp–dextrans with mol. wt $10–80 \times 10^3$.

Below we describe the characterisation of the binding of Gd(III) to pig IgG using PRE and the effects on the PRE probe of the addition of antigens.

2. Theory

The observation of an enhanced solvent water proton relaxation in macromolecule/paramagnetic ion solutions as a result of the binding to the macromolecule is the essence of the PRE technique (reviewed in [6]). As the observed enhancement is proportional to the amount of metal bound to the macromolecule, the technique [3] can be used to yield the metal binding parameters: n, the number of metal binding sites; K_d, the dissociation constant; ϵ_b, an enhancement parameter for each site. The latter is a complex function of a number of molecular motion parameters [6]. Another way of expressing the above is to say that a given paramagnetic relaxation rate is dependent on n, K_d and ϵ_b so that changes in observed rate(s) on perturbing a system, e.g., the addition of an antigen to an antibody, should result from changes in any or a combination of these 3 parameters.

3. Materials and methods

3.1. *Preparation of antibody and antigen solutions*

The following porcine antibodies and antibody fragments were prepared as described: non-specific

IgG, precipitating anti-Dnp IgG antibody, non-precipitating anti-Dnp IgG antibody [7]; non-specific Fab fragment and Fc fragment [8]. The freeze-dried proteins were dissolved in 0.15 M NaCl (BDH Chemicals Ltd, Poole), 50 mM Pipes buffer (BDH) and adjusted to pH 5.5 with 5 M sodium hydroxide. Protein concentrations were determined by absorbance ($A_{1\ cm}^{1\ mg/ml}$ = 1.4 at 280 nm).

Solutions of Gd(III) and protein·Gd(III) were prepared as in [4].

The following antigens with a Dnp group(s) attached to various molecular weight dextrans were prepared as described: mono-Dnp–dextrans of mol. wt 10, 20 and 40×10^3 [9]; Dnp_{14}–dextran of mol. wt 80×10^3 [10]. The antigens were dissolved in the same buffer as for the antibodies.

3.2. *Proton relaxation measurements*

Proton longitudinal and transverse relaxation times were measured on a Bruker B-Kr-322s pulsed spectrometer interfaced with a Varian V-71 computer. Longitudinal relaxation times were measured using a $180°-\tau-90°$ pulse sequence and transverse relaxation times by the Meiboom-Gill modification of the Carr-Purcell sequence.

4. Results and discussion

4.1. *Metal binding studies*

The binding of Gd(III) to the fragments Fab and Fc was studied by PRE using P- and M-titrations as in [3]. The binding parameters obtained are presented in table 1. These parameters can be used to calculate water proton relaxation rates for Gd(III)/porcine whole IgG solutions, assuming that the parameters for the fragments are unchanged in the intact molecule. Such calculated rates are of the order of 30% lower than the observed rates showing that the approximation described, although by no means strictly correct, is likely to be useful to obtain an approximate distribution of metal between bound and free sites in a given solution. For the studies in the presence of antigen the experimental conditions described below were generally such that Gd(III) was expected to be bound to a significant extent to both Fc and Fab sites. The metal ion is therefore reporting on changes in either of these regions on antigen binding.

Table 1
Parameters for the binding of Gd(III) to the porcine IgG fragments Fab and Fc (see section 2)

	Fragment	
	Fab	Fc
n	1	2
K_d (mM)	0.5	0.2
ϵ_b	7	5

The titrations giving these parameters were carried out at a proton resonance frequency of 61 MHz and 19 ± 1°C

Water proton relaxation rates for solutions of Gd(III) and non-specific IgG, non-precipitating and precipitating anti-Dnp antibody under identical conditions were found to be identical within experimental error, suggesting that the Gd(III) binding sites in the 3 antibody species are identical.

4.2. *Relaxation behaviour of non-precipitating antibody in the presence and absence of antigen*

The water proton relaxation behaviour of a Gd(III)/non-precipitating antibody solution in the presence and absence of the Dnp_{14}–dextran antigen was compared over 0–30°C at 61 MHz and 84 MHz. The experimental conditions were such that, using the parameters of table 1, ~35% of the total Gd(III) is estimated to be bound to the Fc sites, ~15% to Fab and ~50% free.

Although non-precipitating at physiological pH, the antibody yielded detectable precipitate at low antigen to antibody stoichiometric ratios at pH 5.5 and the relatively high concentration (10 mg/ml) necessary for PRE studies. The measurements were therefore carried out with the saturate complex, i.e., in the ratio, 1 antigen molecule to 1 antibody binding site.

The relaxation comparison for non-precipitating antibody in the presence and absence of antigen at 84 MHz is presented in fig.1. The rates in the presence and absence of the multivalent antigen are seen to be identical within experimental error (~±5%). A similar effect is observed at 61 MHz. Thus the presence of antigen in the antibody combining site does not produce any changes in antibody structure to which the Gd(III) PRE probes are sensitive.

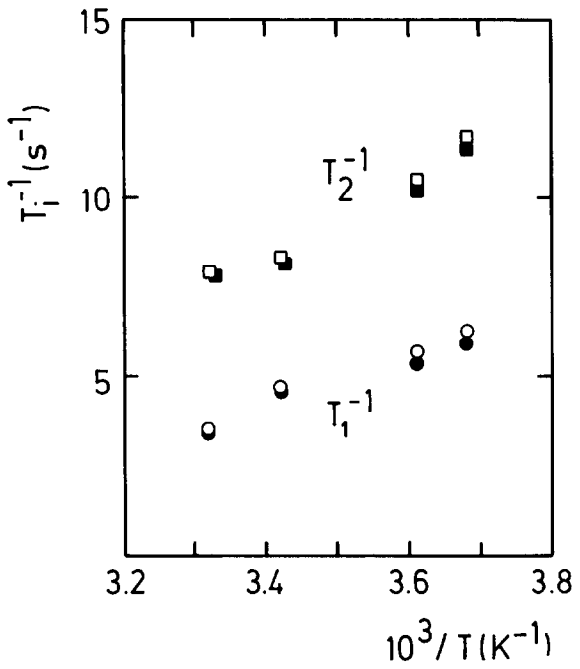

Fig.1. A comparison of the temperature dependence at 84 MHz of the water proton relaxation rates for a solution of non-precipitating porcine IgG and Gd(III) in the presence and absence of a multivalent antigen. (○) T_1^{-1}; (□) T_2^{-1}. Absence of antigen. IgG was 75 μM; Gd(III), 98 μM; 0.15 M NaCl; 0.05 M Pipes buffer (pH 5.5). (●) T_1^{-1}; (■) T_2^{-1}. Presence of Dnp_{14}–dextran antigen, 150 μM. Other conditions as for absence of antigen.

This leads naturally to a consideration of the factors to which the probes are likely to be sensitive. These can be subdivided into the parameters n, K_d and ϵ_b. It seems likely that any changes in n in the presence of antigen for either Fc or Fab sites would produce measurable changes in relaxation rates, and such changes can probably be ruled out. The experimental conditions, ~50% Gd(III) free and ~50% bound, should also allow significant changes in K_d to be observed although small changes, particularly for binding to the Fab region, could produce effects in the water proton relaxation behaviour too small to be detected.

ϵ_b is a more complex parameter depending on the number of exchangeable water molecules coordinating the ion (q), a dipolar correlation time (τ_c) and possibly on the lifetime of a water molecule in the ion sphere (τ_M), depending on the prevailing exchange conditions. Changes in q for either sites on antigen binding would probably lead to large changes in K_d which should be reflected in changes in relaxation behaviour. It is very difficult to say anything about τ_c or τ_M owing to the fact that 'ϵ_b' is a composite of effects from Fc and Fab sites. It would seem likely that radical changes in τ_c or τ_M for either sites would produce measurable effects.

Comparative relaxation studies were also carried out in the presence and absence of mono-Dnp–dextrans of mol. wt 10, 20 and 40×10^3. Again no differences in relaxation behaviour were observed.

4.3. *Relaxation behaviour of precipitating antibody in the presence and absence of antigen*

The water proton relaxation behaviour of a Gd(III)/precipitating IgG solution was compared to that of a number of Gd(III)/precipitating IgG/Dnp_{14}–dextran antigen solutions of varying antigen to antibody ratio over 0–30°C at 61 MHz and 84 MHz. Using the parameters of table 1 it is estimated that ~35% of the total Gd(III) was bound to the Fc sites, ~15% to Fab and ~50% free under the conditions used.

Figure 2 shows the effect of varying antigen to

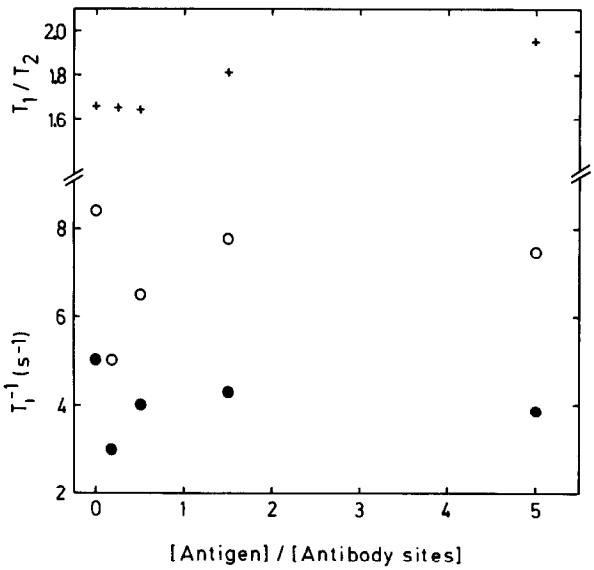

Fig.2. The dependence of water proton relaxation rates on multivalent antigen/antibody ratio at 84 MHz for solutions of precipitating porcine IgG and Gd(III). (○) T_1^{-1}; (●) T_2^{-1}; (+) T_1/T_2. IgG was 75 μM; Gd(III), 98 μM, temp. 19 ± 1°C. Other conditions as in fig.1.

antibody sites ratio at 84 MHz and 19°C, but the trends shown are followed at all temperatures and both frequencies measured. Precipitation becomes visible at antigen to antibody molar ratios lower than one when the relaxation rates are considerably lower than in the absence of antigen, but significantly the T_1/T_2 ratio is left unchanged from that of antibody alone (fig.2). This probably indicates that the decreased rates simply arise from precipitation of antigen–antibody complexes, removing Gd(III) from solution. In high antigen excess where there is no visible precipitation, on the other hand, an increase in the T_1/T_2 ratio of ~10–20% as compared to the uncomplexed antibody is observed. Suitable controls showed that this increase was not due to Gd(III) binding to antigen. Thus some changes in antibody conformation are implied at high antigen excess. We note here the lack of correlation between the observed changes in the T_1/T_2 ratio and complement fixation since the latter is expected to be most efficient at low antigen to antibody ratios.

Relaxation measurements on the precipitating anti-Dnp IgG preparation showed no measurable differences in the presence or absence of mono-Dnp–dextran antigens, as with the non-precipitating IgG.

5. Conclusions

There are, with one exception, no detectable changes in the characteristics of Gd(III) used as a PRE probe located in the Fc and/or Fab region following the recognition of antigen by the antibodies in this work. The exception is for the case of precipitating antibody in the presence of a high excess of multivalent macromolecular antigen, i.e., at full saturation of antibody binding sites. This change is not likely to be functionally related to the capacity of fixing complement, since there is generally very small, if any, complement fixation in high antigen excess. The large differences in conditions for the PRE and complement fixation studies, however, prevent unequivocal conclusions from being drawn. The change seen for antibody saturated with a macromolecular antigen is a novel observation that needs further study to find out whether the phenomenon is generally valid or holds only for some antibody subclasses.

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