SPECIFIC SPIN LABELLING OF THE Fc REGION OF IMMUNOGLOBULINS

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1. Introduction

The binding of antigens to the combining site of immunoglobulins, which is in the Fab region, results in the activation of functions which are located in the Fc region [1,2]. There has been much interest in the mechanism of this activation, but the only direct evidence for a conformational change in the Fc region on antigen binding comes from studies using circular polarisation of luminescence, e.g. [3]. In these studies additional spectral changes are observed when antigen binds to intact IgG compared with the $(Fab')_2$ fragment. However, the evidence for such a conformational change would be more conclusive if the Fc region could be monitored in whole IgG, and this can only be done if a spectroscopic probe can be specifically attached to the Fc region. For example, Gd (III) binds much more tightly to the Fc than to the Fab region of rabbit IgG [4] so that Gd (III) can be used to probe the conformation of the Fc region [5].

The major oligosaccharide present in rabbit IgG is attached to an asparagine residue, which is at position 297 (using the numbering system of the human IgG1 myeloma protein Eu) in the C_H2 region [6]. In this report we show that this carbohydrate can be specifically spin labelled. The environment of this probe can then be studied by electron spin resonance (ESR).

2. Materials and methods

2.1. *Conditions of labelling*

Oxidation of the IgG carbohydrate was carried out at room temperature by addition of sodium periodate, to give a final concentration of 3 mM, to IgG (typically 50 μM) dissolved in 0.03 M sodium acetate buffer, pH 5.5, containing 0.15 M NaCl. Since this oxidation was found to be slow, as followed by subsequent reaction of the oxidised protein with spin label, the oxidation reaction mixture was incubated for 8 h at room temperature. Excess periodate was then removed by extensive dialysis against 0.03 M potassium phosphate buffer, pH 6.0, containing 0.15 M NaCl. 4-Amino-2,2,6,6-tetramethylpiperidin-1-oxyl free radical dissolved in ethanol was added in 25-fold molar excess to the oxidised IgG, and the resulting imine reduced by addition of sodium cyanborohydride to give a 40-fold molar excess with respect to the IgG. No increase in the amount of spin label bound to the protein was obtained by using a higher molar excess of these reagents. The reaction mixture was incubated at room temperature for 6 h, after which time no further increase in the intensity of the ESR signal from covalently bound spin label was observed. Excess reagents were then removed by extensive dialysis against 0.05 M Tris buffer, pH 7.4, containing 0.15 M NaCl. The ESR spectrum of the final dialysate did not show the presence of any remaining non-covalently-bound spin label.

2.2. *Neuraminidase digestion*

Neuraminidase (500 U) was added to non-immune rabbit IgG (50 mg in 5 ml 0.05 M sodium acetate buffer, pH 5.5, containing 0.15 M NaCl and 0.01 M $CaCl_2$), and the mixture incubated at 37°C for 40 h. A control sample of IgG, to which no enzyme had been added, was also incubated under the same

conditions. The IgG was then separated from any free sialic acid by passing down a G-25 column (40 × 2 cm), equilibrated with the same buffer. The protein-containing fractions were pooled and the protein content determined by ultraviolet absorption ($A_{280\,nm}^{1\,mg/ml} \doteq 1.5$). Sialic acid remaining bound to the IgG was determined by the method of Warren [7], after hydrolysis by 0.05 M sulphuric acid (78°C, 1 h).

2.3. *Fluorescence titrations*

Fluorescence quenching titrations of rabbit anti-Dnp antibody were carried out by the addition of aliquots of ε-Dnp-lysine to 1 μM protein solutions, at pH 7.4, using a Perkin-Elmer/Hitachi MPF-2A spectrofluorimeter. An excitation wavelength of 290 nm, emission wavelength of 340 nm, and spectral band-widths of 4 nm were used.

2.4. *Polyacrylamide gel electrophoresis*

Disc electrophoresis was carried out in 7% polyacrylamide gels containing 1% sodium dodecyl sulphate (SDS) [8]. Electrophoresis was done, at pH 7.4, in 0.04 M Tris-acetate buffer, at room temperature, using 6 mA/gel. Protein was stained with Coomassie Blue in methanol/acetic acid/water (5:1:5 v/v/v). Protein samples were reduced in 0.02 M dithiothreitol containing 0.1 M Tris-acetate buffer, pH 8.0, 4 M urea and 1% SDS. Alkylation was carried out with 0.02 M iodoacetamide in the same buffer.

2.5. *Ultracentrifugation experiments*

Sedimentation-velocity experiments were performed on a Beckman model E analytical ultracentrifuge, using Schlieren optics. The runs were carried out, at 20°C, at 52 600 rev/min.

2.6. *Electron spin resonance*

ESR spectra were recorded on a Varian/109 (E-line) spectrometer operating at X-band (9.5 GHz) at room temperature (20°C). Samples were contained in a flat aqueous cell with a syringe attachment for mixing during titrations. A power level of 60 mW and modulation amplitude of 0.2 mT were used. The spin labelled protein concentration was 40–100 μM.

2.7. *Proteins*

IgG was prepared from non-immune rabbit serum [9] and the Fab and Fc fragments prepared by papain digestion as described by Porter [10]. Anti-Dnp serum was prepared by injecting rabbits with Dnp-BGG [11] and the antibody purified by elution from a Dnp-lysine–Sepharose column with 0.1 M 2,4-dinitrophenol in 10 mM Tris buffer, pH 7.4, containining 0.15 M NaCl. Unbound 2,4-dinitrophenol was removed on a G-25 column (60 × 3 cm) and the bound 2,4-dinitrophenol displaced by dialysis against 0.1 M 4-nitrophenol in 0.02 M Tris buffer, pH 8.0, containing 0.15 M NaCl. This hapten was then removed by passing the protein down a Dowex 1X8-400 column (4 × 2 cm), equilibrated with the same buffer. Fluorescence titration with Dnp-lysine showed 1.5–1.7 binding sites on each antibody molecule, with an average association constant of 10^8 (M^{-1}), assuming a mol. wt 150 000 and $A_{280\,nm}^{1\,mg/ml}$ 1.5 for the anti-Dnp antibody. Bis-dinitrophenyloctamethylenediamine was prepared as described by Hyslop et al. [11].

2.8. *Materials*

2,4-Dinitrophenol was obtained from Harrington Brothers Ltd, Weir Road, London SW12 and recrystallised from ethyl acetate. 4-Nitrophenol was obtained from BDH Chemicals Ltd, Poole, Dorset, England and recrystallised three times from hot water. ε-(2,4-Dinitrophenyl)-L-lysine, sodium periodate and sodium arsenite were also obtained from BDH Chemicals, Ltd. Thiobarbituric acid, sodium cyanoborohydride and 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl free radical were obtained from Aldrich Chemical Co. Ltd, Gillingham, Dorset SP8 4JL. Neuraminidase from *Vibrio comma* (cholerae) was obtained from Behringwerke AG, Marburg, FRG. Protein A from *Staphylococcus aureus* was a generous gift of J. Sjödahl, The Biochemical Centre, University of Uppsala, Sweden, as also was a monovalent fragment of protein A [12].

3. Results and discussion

The ESR spectrum of rabbit IgG spin labelled as described above is shown in fig.1, together with the spectra obtained by labelling the isolated Fab and Fc fragments. The intensity of the signal from the spin labelled Fab is less than 10% of that observed with the Fc fragment. Incubation of whole IgG, which had not been oxidised with periodate, with 4-amino-

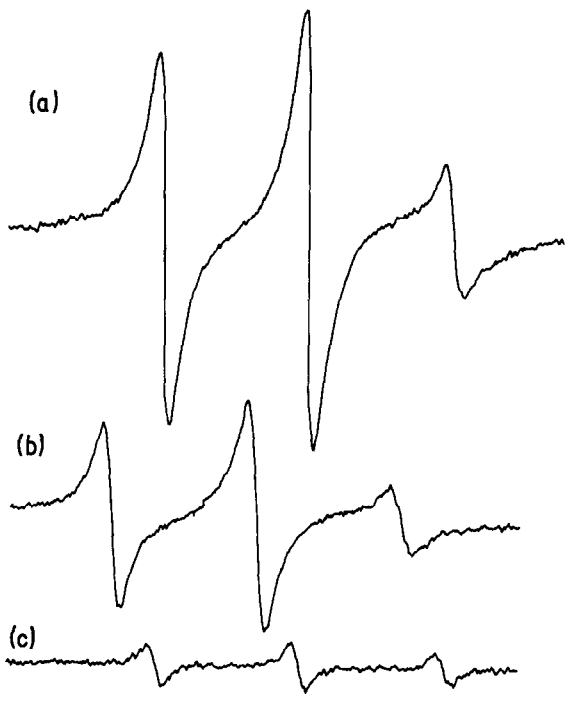

Fig.1. The ESR spectra of: (A) rabbit IgG, 48 μM, (B) Fc fragment, 32 μM and (C) Fab fragment, 39 μM, treated with periodate and spin-labelled as described in the text. Spectra were recorded at 20°C in 0.05 M Tris buffer, pH 7.4, containing 0.15 M NaCl.

2,2,6,6-tetramethyl-piperidin-1-oxyl free radical and cyanoborohydride did not give any observable spin labelling. It is probable that the groups reacting with the spin label are produced by periodate oxidation of the oligosaccharides in the Fc region, and this can be shown to be at least partially true by the removal of the terminal *N*-acetylneuraminic acid residues from the oligosaccharides by neuraminidase digestion. Removal of 82% of the neuraminic acid decreases the intensity of the ESR signal obtained on subsequent spin labelling of the neuraminidase-treated IgG to 48% of that observed with normal IgG, indicating that approximately 60% of the spin label in the untreated IgG is attached to neuraminic acid. This may be due to the known susceptibility of the exocyclic hydroxyls of sialic acid to periodate oxidation [13].

The observed ESR spectrum of the spin labelled IgG is typical of that obtained with a fairly mobile spin label [14]. Huber et al. [15] have shown by X-ray crystallography that the carbohydrate lies between the two C_H2 domains, where it would be expected to be immobilised. The observed mobility if the spin label is consistent with it being on the terminal sugar residues, mainly *N*-acetyneuraminic acid. Polyacrylamide gel electrophoresis of normal and spin labelled IgG shows that the inter-heavy chain disulphide bridge is not broken by the spin labelling treatment.

3.1. *Effect of antigens on the spin label*

The binding of ε-Dnp-lysine to rabbit anti-Dnp antibody is unaffected by the spin labelling of the Fc region, since fluorescence quenching titration still indicates 1.6 binding sites/antibody molecule, with an average association constant of 10^8 (M^{-1}). However, the addition of ε-Dnp-lysine up to equivalence does not result in any changes in the ESR spectrum of the spin label in the Fc region. This observation is consistent with much other evidence which indicates that no large conformational changes occur when small haptens bind to immunoglobulins [16].

The effect of limited aggregation by antigen was studied by the addition of a bivalent hapten, bis-Dnp-octamethylenediamine. Ultracentrifugation studies at antibody-hapten equivalence revealed a population of cross-linked antibody molecules, consisting of about 50% dimers, with higher aggregates and a small peak in the monomer position. However, no change in the ESR spectrum was observed on addition of this hapten to spin labelled anti-Dnp antibody. It is known [11] that only aggregates larger than trimers are capable of complement fixation in this system, and a difference in conformation between the Fc of these aggregates and unliganded monomeric antibody cannot be ruled out, due to the low percentage of these large aggregates in the samples.

Limited aggregation of IgG can also be brought about by protein A, a cell wall protein from *Staphylococcus aureus*. This binds to the Fc region of rabbit IgG [17] leading to the formation of soluble aggregates which can be demonstrated by ultracentrifugation. Neither protein A, nor a monovalent fragment of protein A, affect the ESR spectrum of spin labelled non-immune rabbit IgG, although the spin labelled IgG is still capable of complement fixation in the presence of protein A.

Binding of Dnp-BGG to the spin labelled anti-Dnp

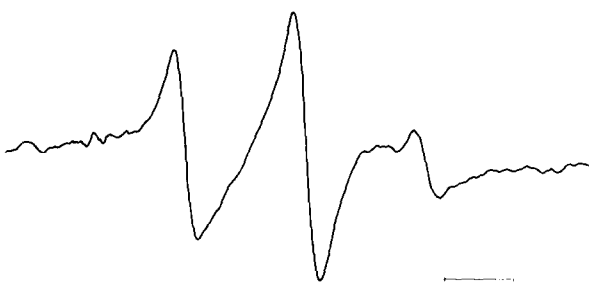

Fig.2. The ESR spectrum of spin-labelled rabbit IgG, 25 μM, in the presence of Dnp-BGG (3 Dnp-groups/antibody molecule). Other conditions as in fig.1.

antibodies can be demonstrated by the presence of an ESR signal in the antigen–antibody precipitate but not in the supernatant. The ESR spectrum of a suspension of the immune precipitate (3.0 Dnp-groups/antibody molecule) is shown in fig.2. Although the spectrum is somewhat broader than that of the monomeric spin labelled antibody, it is remarkable that the spectrum is still characteristic of a spin label with considerable mobility, indicating that the spin label attached to the terminal portion of the oligosaccharide in the C_H2 region is free to rotate independently of the protein. This suggests that on binding Dnp-BGG there is no large conformational change involving the C_H2–C_H2 domain interface, as analysed by probing the environment of the carbohydrate.

Spin labelling of the carbohydrate in the C_H2 domains of immunoglobulins may also be useful as a probe of the effector functions of the Fc region, such as Clq binding and interactions with membranes. This technique may be a general method for labelling glycoproteins specifically at sialic acid residues.

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