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REVIEW

The Dynamics of Glycan-Protein Interactions in Immunoglobulins. Results of Spin Label Studies

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Introduction

The spin-label method developed by McConnell and co-workers [1] is widely used for the study of protein structure in solution. ESR Spectra provide information on the local environment of the label as well as on the general structure of protein molecules [2]. When complex proteins are under study, this approach can be used for elucidation of steric relationships between protein and non-protein parts of the molecule.

One of the most important classes of complex proteins are glycoproteins, which contain covalently bound oligosaccharide chains (glycans). Immunoglobulins (Ig's) also belong to this class of molecules. The sequences of numerous IgG polypeptide chains have been determined, the IgG domain structure has been confirmed, and the principles of cross architecture of IgG molecules are understood (for review see [3, 4]). Therefore, the Ig molecule is a good model for investigating the molecular aspects of protein functions and the role of associated glycans in these functions. Theories on the general physical and biological properties of glycans are currently being developed [5, 6], and the importance of these glycans with respect to both molecular biology and pathology should also be delineated. Indeed, the specificity of many natural polymers is written in terms of sugar

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Abbreviations: TEMPO amine, 4-amino-2,2,6,6-tetramethyl-piperidine-1-oxyl; TEMPO-DT, 4-(*N*-dichlorotriazine)-amino-2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPO-IA, 4-(iodoacetamido)-2,2,6,6-tetramethylpiperidine-1-oxyl; $2A'_{zz}$, the distance between outer wider extremes of the immobilized ESR spectra; 2A, $2A'_{zz}$ of ESR spectra of completely immobilized macromolecule (the spin label is still mobile); $2A_{zz}$, $2A'_{zz}$ of the rigid limit of ESR spectrum (the spin label is highly immobilized); H'(-1), the distance between the high-field outer wide extremes and the central line of ESR spectra; Ig, immunoglobulin; Fab and Fc, proteolytic fragments of immunoglobulin molecules; ESR, electron spin resonance, τ, rotational correlation time.

Table 1. Spin label/protein molar ratio for glycoproteins spin-labeled in the carbohydrate moiety.

Protein	Molar ratio spin-label/protein	Reference
IgG, human	1.62	10
Fab, human IgG	0.3	10
IgG, rat	1.70	10
IgG, pig ani-DNP	1.34	10
IgG, rabbit, pooled	1.54	10
IgG, rabbit, d11	2.0	38
IgG, rabbit, d12	4.0	38
IgE, human	8.0	40
IgG, human	1.80	30
Fc, human IgG	2.2	30
IgG, pig anti-DNS	1.0	53
IgM, human	30.0	40
Fc_5', human IgM	10.0	40
IgA_2 (dimer), human	13.0	41
IgA_1 (monomer), human	4.0	41
Glycophorin	1.2	12
Transferrin	5.0	13

residues rather than amino acids or nucleotides [7]. With this in mind the studies on the special structure of glycans by means of modern physical techniques are of great importance for a better understanding of the molecular mechanisms of their action.

The ESR method is a powerful tool for structural studies of glycoproteins in solution. With that purpose spin labels which possess groups capable of reacting with one or another amino acid residue are usually used. In these cases, however, the information about spatial relations between sugar residues and the protein part of glycoproteins is not available. In recent years selective spin-labelling of carbohydrates has been developed. An excellent review of the use of spin-labeled carbohydrates in studies of various labeled glycoproteins is given by Gnewuch and Sosnovsky [8]. Spin-labelling of glycoprotein glycans can help elucidate which kind of interactions occur in solution between sugar units and the protein moiety of different glycoproteins.

In this review we concentrate on the quantitative analysis of ESR spectra of various classes of Ig's spin-labeled at the carbohydrate moiety. The data obtained are used to determine the relative mobility of the protein oligosaccharide chains and the flexibility of protein as well. Ig molecules were the first glycoproteins, the carbohydrate components of which were spin-labeled [9-11]. Taking Ig's as a model, a quantitative approach to the study of special relationships of glycan and protein moieties of glycoproteins has been proposed.

Incorporation of the Spin Label into Oligosaccharides

At present the methods for obtaining spin-labeled derivatives of carbohydrate residues are well elaborated. An exhaustive description of the procedures is presented in [8].

In the structural studies on Ig's we have used the following procedure [10, 11]. To a 1% solution of protein in 0.1 M acetate buffer, pH 5.0 with 0.14 M NaCl, sodium periodate was added to a final concentration of 20 mM. The mixture was incubated for 16 h at 4°C, and excess ethyleneglycol added, which was followed by incubation at room temperature for 1 h. After the dialysis at 4°C against 0.05 M sodium phosphate buffer with 0.28 M NaCl, pH 8 (changed three times), spin label 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (TEMPO amine) was added in a quantity (mg) 10 times less than the protein taken.

After the incubation for 15 h at 4°C, sodium cyanoborohydride was added to a final concentration of 0.01 M and the solution was incubated overnight under the same conditions. The mixture was exhaustively dialysed for two days against several changes of sodium phosphate buffered saline, pH 7.3. The rest of the free label was removed by gel filtration on a Sephadex G-25 column equilibrated with buffered saline, and the labeled protein was concentrated by "Amicon" ultrafiltration to 20 mg/ml. Spin-labeled proteins may be frozen and stored at very low temperatures.

The Quantity of Spin Label Bound to Immunoglobulin Carbohydrates

By the described method, immunoglobulins of several classes isolated from sera of different species were spin-labeled. As is shown in Table 1, in most cases less than two spin-label molecules were bound to an IgG molecule. A significantly larger amount of spin labels were incorporated in IgE (8 mol/mol protein) and IgM (about 30 mol/mol protein), which can be explained by a higher content of carbohydrates in IgE and IgM molecules as compared to IgG molecules.

In some cases, a high carbohydrate content in the glycoprotein is not correlated with the amount of the spin label bound to sugar residues. Thus, spin-labeled glycophorin [12], which contains about 60% carbohydrate, has only 1-2 mol of the label per mol of protein, yet five mol of label were found to be bound per transferrin molecule [13] (Table1).

As a rule the number of sugar residues of glycoprotein glycans that can be spin-labeled is more numerous than the number of the bound labels per protein molecule. This may be due to the inability of some sugar residues of the native glycoprotein molecule to be modified. Indeed, the core fraction of *N*-glycans is a rigid structure stabilized by hydrogen bonds. Such a structure does not seem to be accessible to water-soluble low molecular weight compounds used for introduction of the spin-label into glycoprotein carbohydrates. Besides, the non-substituted sugar OH-groups are involved in formation of hydrogen bonds both inside oligosaccharide chains and with amino-acids side chains [14, 15] and therefore cannot be oxidized. Thus, the number of sugar residues to be spin-labeled is determined both by the structural specificity of the oligosaccharide chains and by their spatial relationships with the protein moiety as well.

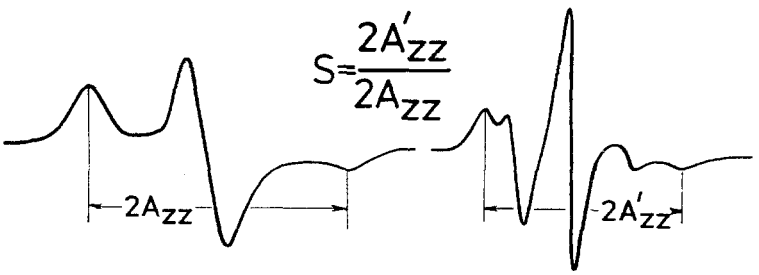

Figure 1. ESR Spectra of the nitroxide radical for the slow-motion realm (right) and for the rigid limit of rotation (left). Distances between outer wide extremes and their ratio (S) are indicated.

ESR Spectra and Their Quantitative Characteristics

A. General Considerations

One of the most important parameters of ESR spectra at slow motional rotation of the nitroxide radical is the distance between the outer wide extremes or the maximum hyperfine splitting ($2A'_{zz}$) (Fig. 1) as well as the ratio (S) of $2A'_{zz}$ to the same distance at the rigid limit ($2A_{zz}$). The value of $2A'_{zz}$ decreases from its limit value (equal to 75 G at 77 K) as the rotation of the spin-label becomes more rapid. Therefore, the less the freedom of the spin-label rotation, the higher is the S value.

After binding to a macromolecule the spin-label can rotate relatively freely or its rotational motion is somewhat restricted. In the former case it is possible to find conditions (for example, to increase the viscosity) in which the macromolecules is immobilised completely while the spin-label is still mobile. In the later case the spin-label is immobilised together with the macromolecule after the solution viscosity is increased. To find out which of these conditions is correct for a given spin-labeled protein molecule, the dependence of $2A'_{zz}$ on the temperature: viscosity ratio should be determined [11, 30]. All the measurements are performed at a constant temperature, and the solution viscosity is changed by addition of increasing amounts of sucrose. If the relative mobility of the spin-label is strongly limited, the isotherm is linear throughout the investigated range of viscosity and intercepts and Y-axis near the rigid limit value, $2A_{zz}$. If the spin-label rotates sufficiently free about the protein molecule, the linear part of the isotherm is found only for small values of viscosity. After a significant rise of sucrose concentration in solution (more than 30% of sucrose), the $2A'_{zz}$ values increase sharply (the "bend" of the isotherm). The linear part of the obtained isotherm is extrapolated to the Y-axis, i.e. extrapolation to infinite viscosity. The closer the point of interception (2A) to the rigid-limit value $2A_{zz}$, the stronger the relative mobility of the restricted label is (for example see Fig. 5).

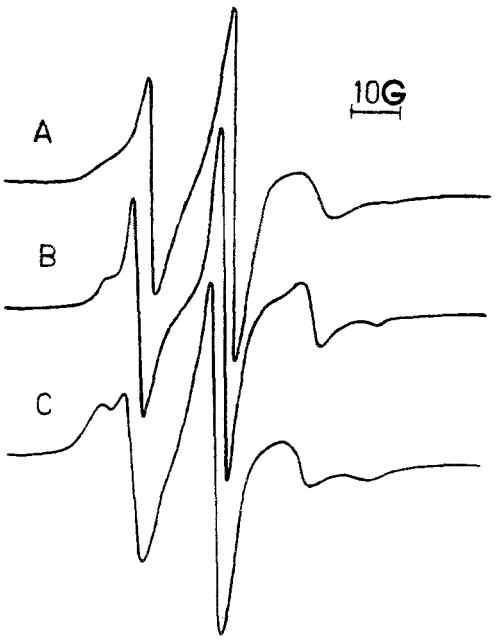

Figure 2. ESR Spectra of human IgG spin-labeled at carbohydrates with TEMPO amine (A) and in the protein moiety with TEMPO-IA (B) and TEMPO-DT (C). 20°C, 0.05 M phosphate buffer with 0.14 M NaCl, pH 7.3.

The slope of the linear part of the isotherm depends on the dimensions of the rotated part of the macromolecule tagged with the spin label: the larger the angle between the linear part of isotherm and the vertical axis, the bigger the rotational unit .

The above spectral parameters are related quantitatively to the rotational motion of the spin-label for values of rotational correlation times τ in the range of 0.1-300 nsec. If rotation of the spin-label is slow ($t \geq 5$ nsec), the shifts of $2A'_{zz}$ from the rigid-limit value $2A_{zz}$ are a quantitative measure of τ and precisely reflect the mobility of the spin label [16]. There are several methods for τ determination of macromolecules, which are based on this correlation [17-23]. The values of τ show whether a macromolecule under study is rigid or flexible. If experimental τ is significantly lower than the calculated one in terms of the rigidity of the macromolecule, then the macromolecule is flexible, i.e. its separate parts are capable of independent relative motion.

B. ESR Spectra of Spin-labeled Immunoglobulins

In Fig. 2, the ESR spectra of IgG labeled with three different covalently bound spin-labels: TEMPO amine (A) TEMPO-DT (B) and TEMPO-IA (C) are presented. There are significant differences between the spectra. Well defined outer wide extremes as well as sharp inner extremes are characteristic for IgG-TEMPO-DT. A possible explanation of the nature of this spectrum is discussed elsewhere [24-28].

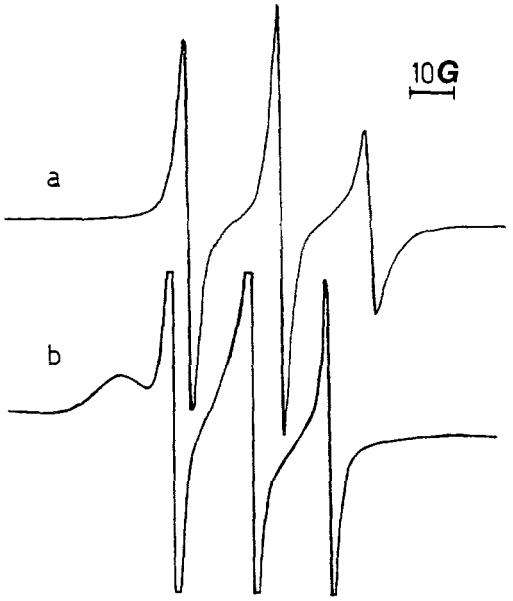

Figure 3. ESR Spectra of pneumococcus polysaccharide spin-labeled with TEMPO amine in the absence (a) and presence (b) of corresponding rabbit homogeneous antibodies. Conditions as in Fig. 2.

ESR spectra of the spin-labeled high-molecular weight polysaccharides are represented by three sharp lines which are close to those characteristic for a spin-label freely mobile in solution (Fig. 3a). This observation is reasonable as isolated polysaccharides are flexible. But ESR spectra of Ig's spin-labeled at carbohydrates reflect restricted rotation of the spin-label (Fig. 2A). This restriction can be explained by close attachment of the oligosaccharide chains together with the bound spin-label to the protein moiety. Our further experiments described below and the studies by other authors support this assumption.

As mentioned above, parameters $2A'_{zz}$ and S characterize quantitatively the rotational mobility of the spin-label; values of $2A'_{zz}$ and S are given in Tables 2 and 3. Analysis of the data presented leads us to the results summarized as follows:

1. The parameters of ESR spectra of Ig's labeled at carbohydrates are higher than the same parameters of Ig's labeled with two other spin-labels at the protein moiety. For example, the S value for IgG tagged at its carbohydrate is equal to 0.81, whereas the S value for the same protein tagged with TEMPO-DT or TEMPO-IA is equal to 0.71.

2. The value of the distance between the outer wide extremes $2A'_{zz}$ is affected not only by the rate of rotational motion of the spin-label but also by the chemical environment of the nitroxide group. But the influence of the latter factor as compared to the former is

Table 2. Parameters of ESR spectra of spin-labeled immunoglobulins (20°C, 0.05 M phosphate buffer with 0.14 M NaCl, pH 7.3).

Spin-labeled immunoglobulin	Label	$2A'_{zz}$	S[a]	Reference
Glycoproteins labeled in the protein moiety				
IgG[b]	TEMPO-DT	53	0.71	11, 30, 52
IgE, human	TEMPO-DT	54	0.72	40
IgM, human	TEMPO-DT	55.5	0.74	36
IgG_1, human	TEMPO-IA	53	0.71	11
IgA_1, human	TEMPO-DT	53	0.71	41
IgA_2, human	TEMPO-DT	55.5[c]	0.74	41
IgG, pig anti-DNS	TEMPO-IA	52	0.69	57
Glycoproteins labeled in the carbohydrate moiety				
IgG, human	TEMPO amine	60.7[d]	0.81	30
Fc, human IgG	TEMPO amine	62.9[d]	0.84	30
IgM, human	TEMPO amine	59.3[e]	0.79	40
IgM_s-subunits, human IgM	TEMPO amine	60.3[e]	0.80	40
Fc_5, human IgM	TEMPO amine	58.3[e]	0.78	40

[a] $S = 2A'_{zz}/2A_{zz}$, where $2A_{zz} = 75$ G (at 77 K).
[b] Rabbit, human, pig anti-DNS, rabbit anti-human IgG and anti-pneumococcus polysaccharides.
[c] $2A'_{zz}$ was measured at 21°C in 0.05 M phosphate buffer, 0.14 M NaCl, pH 7.3.
[d] $2A'_{zz}$ was measured using the plots in Fig. 5 at $T/\eta = 300$ (corresponding to 20°C and 0% sucrose).
[e] $2A'_{zz}$ was measured using the plots given in [38].

relatively small [29]. Differences in the $2A_{zz}$ values of spectra of Ig's labeled with various spin-labels are so large (5-7 G) that they cannot be simply a consequence of the different environment of the NO group. The higher $2A_{zz}$ values of ESR spectra of carbohydrate labeled proteins are likely to be due to restricted rotation of the spin-label.

3. Temperature changes and increases of the solution viscosity affect ESR spectra of spin-labeled Ig's in a different way: the influence of the decreasing temperature or addition of sucrose to the solution are more pronounced for proteins tagged at carbohydrates. For example, after addition of sucrose to 33%, the 2A'zz value for IgM and IgG labeled at carbohydrates increases by 7.7 G and 6.5 G (at 20°C), respectively, while the increase of 2A'zz for the same proteins tagged with TEMPO-DT after sucrose addition is equal only to 1.8 G and 1.0 G.

These data point to a firm attachment of the TEMPO amine together with oligosaccharide chains to the nearby parts of the Ig molecule.

The ESR spectra of Ig's belonging to different classes and labeled with the same spin-label do not differ significantly. For example, the ESR spectra of IgG, IgM, IgA_2 (dimers) and IgA_1 (monomers) labeled at carbohydrates are quite similar (see below). The stability of the shape of the ESR spectra for different classes of immunoglobulins and other glycoproteins (see original papers reviewed in [8]) spin-labeled at carbohydrate components may result from

Table 3. Parameters of ESR spectra of immobilized spin-labeled immunoglobulins (0.05 M phosphate buffer with 0.14 M NaCl, pH 7.3).

Labeled macromolecule	Label	Immobilization	$2A'_{zz}$	S[a]	Reference
Glycoproteins labeled in the protein moiety					
IgG, rabbit	TEMPO-DT	0°C	55	0.74	30
IgM, human	TEMPO-DT	0°C	57	0.76	36
IgG rabbit anti-human IgG and anti-pneumococcus polysaccharides	TEMPO-DT	20°C, soluble complexes with antigen and antigen-antibody precipitate	54	0.72	111
IgG, human, as antigen	TEMPO-DT	20°C, soluble complexes with antibody	53	0.71	11
	or TEMPO-IA	20°C, antigen-antibody precipitate	55	0.74	11
IgG, rabbit anti-TEMPO-DT	TEMPO-DT	20°C, TEMPO-DT in the combining site	67	0.89	57
IgAMOPC-315, mouse	TEMPO-DT	20°C, TEMPO-DT in the combining site	65	0.87	11
Glycoproteins labeled in the carbohydrate moiety					
IgG, human	TEMPO amine	0°C	64	0.85	30
Fc, human IgG	TEMPO amine	0°C	65.8	0.88	30
IgM, human	TEMPO amine	0°C	64[b]	0.85	40
IgM_s subunits	TEMPO amine	0°C	64.5[b]	0.86	40
Fc_5, human IgM	TEMPO amine	0°C	63	0.84	40

[a] $S = 2A'_{zz}/2A_{zz}$, where $2A_{zz} = 75$ G (at 77 K).
[b] 2A'zz was measured using the plots given in [38].

an identical common structure of oligosaccharide chains. Glycans have a double structure - a rigid hydrophobic core unit and loose mobile antennae [6]. The core fraction of glycans performs the role of an anchor. It interacts with the protein moiety of glycoprotein and determines thereby the anisotropic rotation of spin-label bound to mobile antennae. Such character of rotational mobility of spin-labels is reflected by the moderately immobilized ESR spectra. On the other hand, the analysis of the electron density of different sugar residues in the crystals of the rabbit IgG Fc fragment showed the mobility of monosaccharides to be different [15]. Since the sugar residues with different mobility can be spin-labeled, the summarized ESR spectrum seems to represent a superposition of ESR spectra or spin-labels with different mobility. A greater or a smaller degree of immobilization of ESR spectra depends on spatial relations of spin-labeled sugar residues to the protein moiety in each case.

Below we review the structural studies on different Ig's spin-labeled in the carbohydrate moiety.

Immunoglobulin Molecules

A. Immunoglobulin G

Fig. 4 shows the ESR spectra of human IgG and its Fc fragment spin-labeled in its carbohydrate [30]. At normal conditions (20°C and 0% sucrose) these spectra reflect moderately immobilised rotation of the spin-label. The outer wide extremes are poorly resolved. After addition of sucrose and at 0°C, however, the extremes are well seen. This makes it possible to determine the distance between them (2A'zz), which in turn depends on the mobility of the spin-label.

The dependences of $2A'_{zz}$ values on solution viscosity at constant temperature for IgG and Fc spin-labeled at carbohydrates are presented in Fig. 5 (upper plots). Both isotherms are linear in the range of investigated viscosities and their extrapolation gives rise to $2\bar{A}$ values (70-71 G) close to the rigid-limit $2A'_{zz}$. Obviously, the restriction of mobility of a spin-label bound to carbohydrates parallels the restriction of macromolecule rotation. These data favour a close attachment of oligosaccharide chains to the protein moiety.

Fig. 5 (bottom plots) shows that isotherms of IgG, its $(F(ab')_2$ and Fab'_2 fragments spin-labeled with TEMPO-DT in the protein moiety. All three isotherms differ from the isotherms described above, being made up of two parts. One of them is linear and reflects gradual immobilisation of the macromolecule tumbling at relatively low viscosities (the spin-label retains the pronounced mobility). The further increase of viscosity sharply increases the $2A'_{zz}$ values as a consequence of hindrance of the spin-label rotation. The intercept of the linear part of the isotherm with the vertical axis in this experiments is equal to 56.6 G($2\bar{A}$), i.e. significantly lower than the $2A_{zz}$ value (75 G). The spin-label rotation relative to the immobilised macromolecule is characterised by the ($2A_{zz}$-$2\bar{A}$) value, equal to 18.5 G.

To obtain the τ values of IgG and Fc spin-labeled at protein and carbohydrate moieties we have employed a method based on the assumption of slow isotropic tumbling of the macromolecule and a faster anisotropic motion of the spin-label relative to the macromolecule [21-23]. The τ values for IgG, $F(ab)_2$ and Fab tagged by TEMPO-DT are found to be equal to 26,25, and 21 nsec and correspond well to the τ value calculated for a rigid sphere with mol. wt. 50,000 [31, 32]. These results are in close agreement with the values of τ obtained from fluorescence depolarization measurements [31, 32] and confirm the relative freedom of rotation of Fab fragments. Thus, the question about the ability of Fab fragments for significant relative motion [33, 34] is settled.

The τ values for Fc are two times smaller (12 nsec) than the τ value predicted for a rigid sphere with mol .wt. 50,000. This fact suggests the existence of an internal lability of Fc, which seems to be important for its biological activity. Again, the obtained τ values are in good agreement with those found for Fc by fluorescence depolarization [31]. The correlation of data obtained by both methods has also been previously noted for IgE [35] and recently for IgM and IgA [36].

The two major observations of our experiments: close attachment of the carbohydrate component to the protein moiety and the flexibility of Fc are consistent with the X-ray data

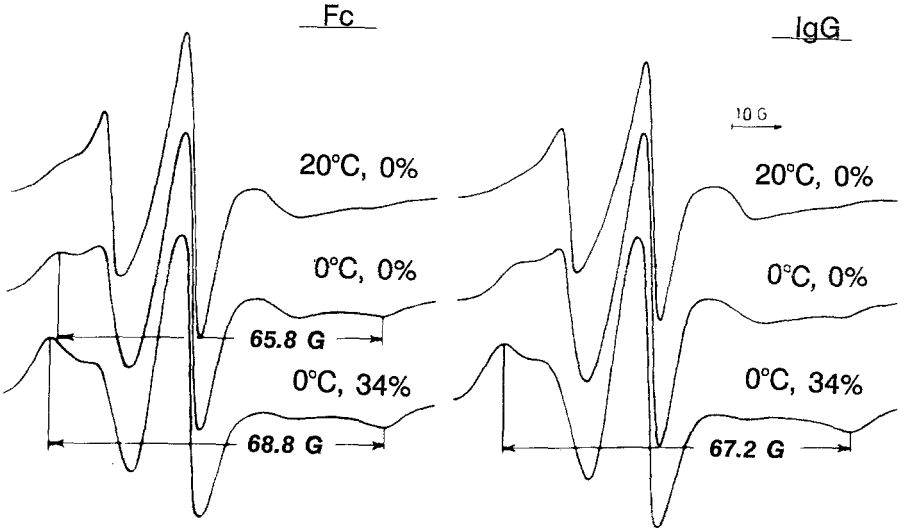

Figure 4. ESR Spectra of human IgG and Fc spin-labeled in the carbohydrate. Conditions are designated for each spectrum in the Figure.

[14]. ^{13}C Nuclear magnetic resonance (NMR) spectra of the Fc portion of IgG_1 have also been investigated [37]. In the NMR spectra, two broad bands are observed due to resonance from carbohydrates. The broadness of these lines suggests a lack of independent mobility of the carbohydrate component relative to the protein moiety.

It can thus be concluded that three different methods, i.e. X-ray crystallography, NMR spectroscopy and spin-labelling, are in good agreement concerning the restriction of independent motion of IgG oligosaccharides chains.

The IgG molecules were spin-labeled at carbohydrate components in other laboratories [9, 38]. In all cases periodate oxidation and subsequent reductive amination were used. The oxidation with periodate was time limited (10-20 min) so that the label could be selectively introduced into the terminal residues of sialic acid. At the same time, it is known that the most IgG molecules (both monoclonal and polyclonal) carry mainly neutral oligosaccharides, and only 20% of IgG glycan's antennae are sialylated [39]. Therefore, a selective introduction of the spin-label into the terminal residues of sialic acid would allow only a small part of IgG molecules in the sample to be labeled. However, the molar ratio of spin-label/protein was found to be equal to 2-4 in these studies [38]. Obviously, not only sialic acid residues were spin-labeled in these experiments but also other sugar residues. The authors [38]

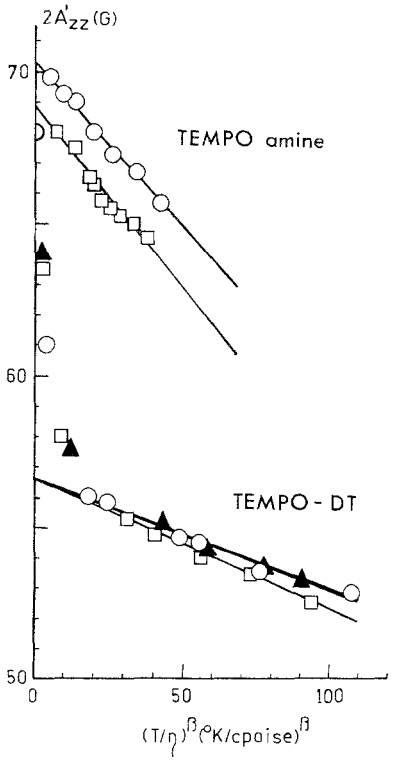

Figure 5. The distance between outer wide extremes ($2A'_{zz}$) of ESR spectra vs (T/η) for:- *upper plots*; human IgG (□) and Fc (○) labeled at carbohydrates ($\beta = 0.74$); *lower plots*, rabbit IgG (○), F(ab')$_2$ (△) and Fab' (□) labeled in the protein moiety with TEMPO-DT ($\beta = 0.82$). Measurements were carried out at constant temperature. The viscosity was changed by adding different amounts of sucrose. Dependences are plotted from a least-squares fit of experimental data.

established the effective values of τ for the spin-labels bound to carbohydrate components of rabbit IgG antibodies both intact and in specific and non-specific immune complexes. On the basis of the τ values obtained it is difficult to judge relative mobility of immunoglobulin domains because in order to calculate the τ value the authors used the isotropic diffusion model for the spin-label rotation whereas the spin-label attached to carbohydrate components is rotated anisotropically. The obtained values (7-13 nsec) rather demonstrate a limited relative mobility of spin-labeled oligosaccharides in the IgG molecule.

B. Other Classes of Immunoglobulins

All classes of Ig's contain carbohydrates. It is of interest whether the interrelations between carbohydrates and the protein moiety found for IgG are the same for other classes of Ig's as well.

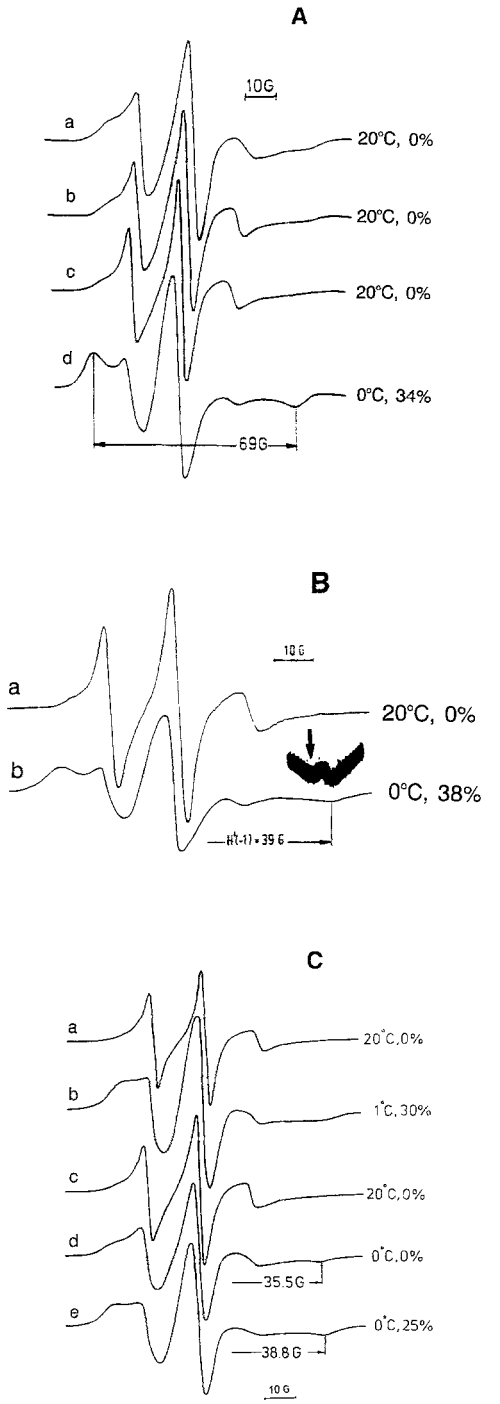

Figure 6. ESR Spectra of immunoglobulins spin-labeled in the carbohydrate. A. (a), (d); IgM human: (b); Fc_5, human IgM: (c); IgM_s-subunits, human IgM. B. (a), (b); IgE human. C. (a), (b); IgA_2 human: (c), (d), (e); IgA_1 human. Conditions are designated for each spectrum in the Figure.

With this in mind, we have investigated the rotational mobility of spin-labels bound to carbohydrates of myeloma immunoglobulins of different classes: IgM, its 7S subunits (IgM_s) and Fc_5 fragment [11, 40], IgA_1 and IgA_2 [41], IgE [40]. The spectra of all these proteins are similar to the spectra of IgG labeled by the same method (Fig. 6). The form of the ESR spectra reflects a restricted rotation of the spin-label. The outer wide extremes are not seen at 20°C and 0% sucrose, but spacings between them ($2A'_{zz}$) are detectable after addition of sucrose and decreases of temperature. It should be noted that the temperature and viscosity influence on the spectra of Ig's described here is different from that seen for IgG. For example, the outer wide extremes of ESR spectra of IgM-TEMPO-amine appear only after sucrose addition of 18% at 0°C (Fig. 6A). At the same time, the extremes of ESR spectra of labeled IgG carbohydrates are observed after the decreasing of temperature alone (without sucrose addition). Obviously, the IgM molecules possess not only carbohydrates units closely attached to the protein surface, but also have units which have a larger freedom of rotation. A different relative mobility of oligosaccharides of the human myeloma IgM has also been found [42, 43].

The ESR spectra of IgE spin-labeled at carbohydrates [40] possess one (or more) additional extreme better seen in the high-field part of the spectra (Fig. 6B, designated by an arrow). Each epsilon chain has six oligosaccharides [44] and probably the spin-label can be bound to either of them (four spin-labels per epsilon chain [40]). The presence of more than one wide extreme in the ESR spectra can be explained by existence of various relative mobilities of different oligosaccharide chains. The additional wide extreme of ESR spectra located near the central line (Fig. 6B) reflects the rotation of the spin-labels bound to oligosaccharides which are more mobile in solution.

The dependence of the distance of the high-field extreme H'(-1) of ESR spectra on $(T/\eta)^{0.67}$ for IgE spin-labeled at carbohydrates is shown in Fig. 7A. This isotherm intercepted the Y-axis at the point near 40 G in a similar manner as the isotherm for the spin-label firmly located in the combining sites of anti-DNP pig antibodies [45]. Evidently, a part of the spin-labels bound to IgE carbohydrates are strongly immobilized due to close attachment of oligosaccharide chains to the protein moiety.

The outer wide extremes of ESR spectra of IgA_1-TEMPO amine are highly heterogeneous (Fig. 6C, [41]). It may be explained by the presence of spin-labels with different degrees of immobilization. These extremes are better resolved in the high-field part of the spectrum. The dependence of H'(-1) value upon solution viscosity at 0°C is plotted in Fig. 7B. This isotherm is identical to that found for IgE-TEMPO-amine. Therefore, at least some of the spin-labels linked to sugar residues of IgA_1 are immobilized simultaneously with the macromolecule. On the other hand, in the IgA_1 hinge region there are five *O*-glycosidic disaccharides [46] that also can be spin-labeled. This region has no regular structure and is flexible. Therefore, hinge oligosaccharides can possess a significant freedom of rotation.

The spin-label linked to IgA_2 carbohydrates generates ESR spectra without distinct outer wide extremes (Fig. 6C [41]). Even after decrease of temperature to 1°C and addition of sucrose to 30%, these extremes were also not resolved. At these conditions the rotation of the IgA_2 molecule is significantly restricted, while spin-labeled sugar residues retain a pronounced mobility. It follows, that the spin-labeled carbohydrate components have a significant relative freedom of rotation and are exposed in solution.

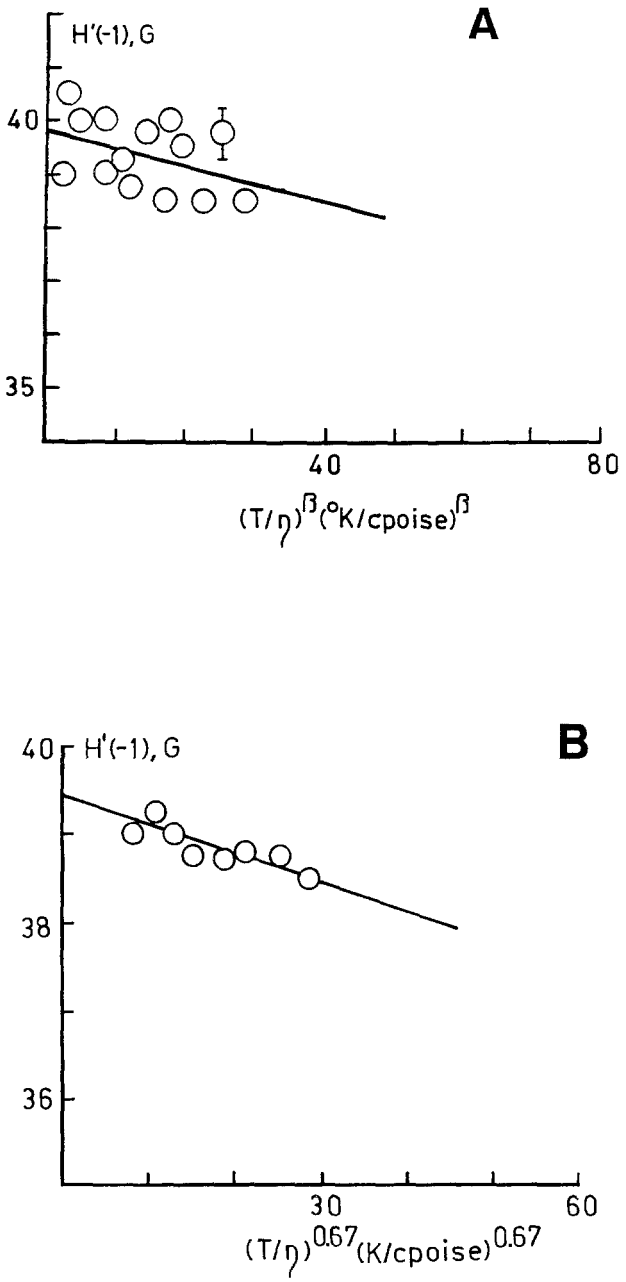

Figure 7. The distance of the high-field extremes H'(-1) from the central line of ESR spectra vs $(T/\eta)^{\beta}$ for IgE(A) and IgA_1(B), labeled in the carbohydrate ($\beta = 0.67$). For details see Fig. 5.

For all spin-labeled immunoglobulins and their fragments we plotted the dependences of the parameters of ESR spectra [2A'zz or $H^1(-1)$] versus viscosity of the solution at constant temperature - isotherms. Typical example of these are given above for IgG, IgE and IgA_1. On the basis of the isotherms the rotational correlation times were calculated. The data obtained are summarized in Table 4. The τ values found for isolated antigen-binding fragments clearly demonstrate the rigid structure of Fab. While the values of τ obtained for Fcγ (12 nsec) and Fc_5 (4-6 nsec) correspond to the τ values calculated for a unit with dimensions of 1-2 domains but are much higher than the τ values of Ig oligosaccharides (mol.wt. 2,000-3,000) if their rotation is free. These data suggest there is internal lability of Fc fragments and that there is restricted motion of Fc-linked carbohydrates. Preliminary results point to the absence of internal flexibility of IgA_1 and IgE Fc fragments. Further experiments with isolated Fc fragments are needed to confirm these provocative data.

Nezlin *et al.* [48, 49] determined the τ-values of sugar-labeled extracellular portions of HLA antigens of class I and class II, which are related to the superfamily of immunoglobulin molecules. In both cases these values were equal to 2.0-2.4 nsec. These data pointed to the restricted rotation of the oligosaccharide chains attached to small mobile domains of HLA molecules.

Studies of the Antigen-Antibody Interaction

A. Hapten-induced Conformational Changes of Antibodies, Spin-labeled in the Carbohydrate Moiety

The results of the studies on ligand-induced structural changes of antibody molecules are controversial for the reason that these changes have not always been observed in all experiments [50]. The conformational changes have been detected by a spin-label technique, in particular, in the Fab region [24, 51]. As recently shown, they are stoichiometrically related to the binding of the monovalent ligand [52]. However, to demonstrate changes in the Fc part is more important as it will demonstrate the transmitting of a signal along the antibody molecule after complexing with the antigen. Usually, selective introduction of a responsive reporter group in the Fc part is one of the main difficulties for such studies. The possibility of specific spin-labeling of Ig carbohydrates partly resolves this problem.

In the first experiments on IgG antibodies spin-labeled at carbohydrates, the ESR spectra before and after formation of the specific complex with antigen were compared [9, 10]. In both cases, no changes have been observed in the patterns of ESR spectra of labeled antibodies after interaction with antigen.

Recently, in addition to these attempts to find qualitative changes in ESR spectra, rotational correlation times of carbohydrate labeled antibodies before and after interaction with the low-molecular weight ligand have been determined [53]. In these experiments, pig anti-DNS antibodies of the IgG class are used. Previously, it has been shown that after ligand binding, the slow conformational changes correlated with the amount of hapten added, and be assigned most probably to the Fab region of these antibodies (TEMPO-DT spin-label [52]). The observed dependences of the reciprocal τ values versus the T/η ratio are different for the antibodies before and after ligand binding.

Table 4. Rotational correlation times, τ, of spin-labeled immunoglobulins and their fragments.

Spin-labeled protein	Label	τ[a] (ns)	Reference
IgG, rabbit	TEMPO-DT	26	30
$F(ab')_2$, rabbit IgG	TEMPO-DT	25	30
Fab', rabbit IgG	TEMPO-DT	21	30
IgM, human	TEMPO-DT	50	36
Fab, human IgM	TEMPO-DT	22	43
IgA_1, human	TEMPO-DT	32, 41	36, 41
IgA_2, human	TEMPO-DT	45	41
IgE, human	TEMPO-DT	60-64	40, 47
IgG, human	TEMPO-amine	11	30
Fc, human IgG	TEMPO-amine	12	30
IgM, human	TEMPO-amine	7-9.5	40, 42, 43
Fc_5', human IgM	TEMPO-amine	4-6	40, 42, 43
IgMs-subunit, human IgM	TEMPO-amine	7	40
IgA_1, human	TEMPO-amine	45	41
IgE, human	TEMPO-amine	66	40

[a] All the τ-values are given for standard conditions (20°C, 0% sucrose).

Thus, in these experiments the changes in the spin-label mobility are a consequence of occupation of the combining sites by hapten molecules. In IgG molecules, the majority of carbohydrates are attached to Fc region, and it is most probable that in pig IgG antibodies they are also located in the same region. Therefore, the observed changes in the spin-label rotation can be associated with the structural changes of the Fc part after hapten binding. Further experiments are necessary to confirm these provocative findings. Particularly, correlation of the observed effect with the amount of added hapten has to be established [50].

B. Reaction of the Spin-labeled Polysaccharide Antigen with Antibodies

ESR Spectra of the spin-labeled high molecular weight polysaccharide antigen isolated from pneumococcus SII are characteristic for the relatively free rotation of the bound spin-label, as separate units of the polysaccharide chain are mobile (Fig. 3). After addition of the labeled antigen to an excess of corresponding rabbit homogeneous antibody, well-separated outer wide extremes appear in the ESR spectra [11]. This points to a restricted mobility of part of the bound spin-labels due to immobilization of a portion of antigen in the antibody combining site. Therefore, by a spin-label method the antigen-antibody reaction can be traced not only for small ligands, but for large spin-labeled macromolecules as well.

Interaction of Spin-labeled Immunoglobulins and Immune Complexes with Other Molecules

Using glycoproteins selectively spin-labeled in the carbohydrate, it is possible to find how closely the contact region involved in complex formation with one or another substance locates to the oligosaccharide. The data obtained in such experiments are more informative

if the position of the oligosaccharides is known. A good example is IgG molecules that have two symmetrical carbohydrate units placed in the CH2 domains (Fc). Complexing the Fc fragment with staphylococcus protein A has no effect on ESR spectra of IgG spin-labeled on oligosaccharides [9; Nezlin RS, Sykulev YK, unpublished data]. Obviously, the contact region on the IgG molecule surface is relatively far from the oligosaccharide. The X-ray data confirm this suggestion [54].

By the same method, the binding of low-molecular weight substances to the Fc fragment has been studied [55]. These substances form complexes with Fc and can be removed from the protein by dialysis at pH 5. It is found that addition of the substances to IgG first dialyzed at pH 5 and spin-labeled in the carbohydrates changes the patterns of ESR spectra. Particularly, the outer wide extremes shift by 2 G. The results obtained point to the formation of a close contact of the substances with the spin-labeled carbohydrates or to conformational changes after complex formation, which influence the region around the spin-label. In other work [38] the authors studied the ESR spectra of rabbit IgG spin-labeled at carbohydrate components in model immune complexes obtained as a result of specific (under the action of antigen) and non-specific (covalent linking of molecules) aggregation of IgG antibodies. The quantitative analysis of the shape of ESR spectra demonstrated that the formation of immune complexes is followed by the limitation of the mobility of the label connected with the IgG carbohydrates. These limitations are of greater extent in the specific immune complexes. The authors believe that in the antibody-antigen complexes, protein-protein (Fc:Fc) interactions occur, and these interactions depend on the molecular masses of the complexes [56]. When adding the C1q component of complement to antigen-antibody complexes, one could observe a considerable immobilization of the ESR spectrum. The 2A'zz value increased by 2-3 G. This effect was not observed for the non-specific IgG complexes. Obviously, C1q causes a definite spatial orientation of Fc fragments in antigen-antibody complexes, which is required for the interaction with C1s/C1r. Upon interaction of C1q with non-specific complexes such spatial orientation of Fc fragments does not occur. It is also known that the covalently linked IgC complexes, unlike specific immune complexes, are not capable of activating the C1 component, although both type of the complexes interact with C1q.

Finally, we note that the approach described herein can be used to introduce other reporter groups (e.g. fluorescent or radioactive) into the carbohydrate moieties of Ig's. The results of studies using such probes are reviewed by O'Shannessy and Quarles [58].

Concluding Remarks

As is shown by the data presented, the spin-label bound to carbohydrate components of immunoglobulins provides valuable information on the spatial location of oligosaccharide chains as well as on the general structure of Ig molecules. In the course of the studies on spin-labeled immunoglobulins, valuable data have been obtained for IgG molecules. It is shown that rotation of spin-labels bound to carbohydrates is strongly restricted. The most probable reason for the spin-label immobilization is close attachment of oligosaccharide chains to the protein moiety in solution. According to the observed value of the rotational correlation times, the Fc fragment has a flexible structure.

Heavy chains of IgM, IgE and IgA have several oligosaccharide units per heavy chain. As it is shown by the spin-label approach, in IgM, IgE and IgA_1 molecules along with oligosaccharides that have a significant freedom of rotation, there exist oligosaccharides attached tightly to the protein moiety. More probably, all oligosaccharides of IgA_2 molecules can rotate freely. Thus immunoglobulins possess two types of carbohydrate units which are different in their spatial interrelations with the protein surface.

The study of the interrelation of carbohydrate units with the protein surface of glycoproteins is important for understanding their structural features and biological properties. If carbohydrate residues are tightly attached to the protein surface, they can shield some amino-acid residues, thus interfering in this way with the contacts between polypeptide subunits (as in Fc of IgG) or preventing the action of proteolytic enzymes. Carbohydrate residues which are not fixed could participate in recognition and specific binding of different biologically-active substances, for example, of cell membranes.

In spite of some methodological problems, the spin-labeling of glycoprotein glycans allows one to study spatial interrelationships between sugar residues and the protein moiety of glycoproteins. This approach is also useful for elucidation of a functional role of carbohydrates of these molecules. For example spin-labeling of the IgG carbohydrates can also be helpful for investigation of antibody effector functions such as interaction with different Fc receptors and fixation of immunoglobulins on biological membranes.

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