ROTATIONAL CORRELATION TIMES OF IgG AND ITS FRAGMENTS SPIN-LABELED AT CARBOHYDRATE OR PROTEIN MOIETIES

Spatially fixed position of the Fc carbohydrate

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1. Introduction

The values of the rotational correlation time (τ) of a macromolecule gives information whether its structure is rigid or flexible. For example, the τ values of immunoglobulin molecules, which are considerably lower than the τ values calculated assuming rigidity of these proteins, point to the existence of relatively independent rotation of Fab fragments bearing the antibody combining sites [1]. To determine τ of macromolecules the dependence of the distance between outer wide extrema of immobilized ESR spectra of spin-labeled proteins on τ have been used [2–7].

Recently the possibility of spin-labeling of immunoglobulin carbohydrates was shown [8,9]. This approach allows for the first time investigation in solution of the dynamic behaviour of carbohydrate chains of glycoproteins. In the present study we determined the τ values of IgG and its Fc fragment spin-labeled at carbohydrates by means of the method, which permits evaluation of the rotation of spin-label relative to a macromolecule [7]. The data obtained provide direct evidence for the existence of an internal lability of Fc fragment and for the rigid fixation of its carbohydrate component in solution.

Abbreviations: τ, rotational correlation time; $2A'$, distance between outer wide extrema of immobilized ESR spectrum; $2A_{\tau}$, the $2A'$ of completely immobilized spin-label; $2\bar{A}$, the $2A'$ at complete immobilization of macromolecule; IgG-SLI and Fc-SLI, IgG and Fc labeled at carbohydrates with spin-label SLI; IgG-SLII, $F(ab')_2$-SLII and Fab'-SLII, IgG and $F(ab')_2$ and Fab' labeled with spin-label SLII

2. Materials and methods

2.1. *Proteins and their spin-labeling*

Human and rabbit IgG were isolated from commercial preparations of gamma-globulins by ion-exchange chromatography on DEAE-cellulose in 0.02 M phosphate buffer, pH 6.3. Spin-labeling of human IgG and papain Fc carbohydrates was performed after periodate oxidation as in [8] with some modifications: firstly, the amount of the added spin-label SLI (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl, Aldrich Chem. Co.) was 10-times less and, secondly, before gel-filtration on Sephadex G-25 the spin-labeled proteins were dialysed exhaustively for more complete removal of unreacted spin-label.

Rabbit IgG and its peptic fragments were labeled with spin-label SLII (2,2,6,6,-tetramethylpiperidine-4-amino-(*N*-dichlorotriazine) in 0.05 M phosphate buffer, pH 7.3 for 3 days at 4°C. In IgG-SLII the spin-label was found only on Fab [1]. The amount of bound spin-labels was determined by double integration of ESR spectra.

2.2. *Determination of rotational correlation time*

The determination of the τ value and the evaluation of relative rotational freedom of spin-labels are based on the following considerations [3,4,7]. The value of $2A'$ of ESR spectra decreases as spin-label motion becomes more rapid. If spin-label is fixed rigidly to a protein molecule, $2A'$ depends only on the τ value of macromolecule [3]. But if macromolecule is completely immobilized, $2A'$ reflects only the rotation of spin-label ($2A'=2\bar{A}$). In general $2A'$ depends

on the motion of macromolecule as well as on the relative motion of spin-label. Hence, the overall shift $(2A_z - 2A')$ can be expressed as:

$$2A_z - 2A' = (2A_z - 2\bar{A}) + (2\bar{A} - 2A')$$

where $(2A_z - 2\bar{A})$ is a shift due to the relative motion of the spin-label and $(2\bar{A} - 2A')$ is a shift due to the rotation of the macromolecule.

The ESR spectra of a nitroxide radical were simulated on a computor assuming the slow isotropic Brownian diffusion of the macromolecule [10] and rapid anisotropic motion of spin-label relative to macromolecule [5,7]. The shift $(2\bar{A} - 2A')$ of these simulated spectra was proportional to $\tau^{-\beta}$ where β depends on the relative motion of spin-label [7].

To determine β, the value of $2A'$ was measured at 0°C and 45% sucrose concentration. The β was found for the obtained value of $2A'$ (approximately equal to $2\bar{A}$ under these conditions) using the nomogram (fig.4 in [7]). For human IgG-SLI and Fc-SLI β was equal to 0.74 and for rabbit IgG-SLII and its fragments labeled with SLII the $\beta = 0.82$. The $2A_z$ determined at 0°C and 68% sucrose concentration was found to be equal 71 ± 1 G.

The ESR spectra of the spin-labeled proteins were run at constant temperatures and increasing sucrose concentrations (up to 45%) on a E-104A Varian spectrometer. Since $\tau \propto \eta/T$ the values of $2A'$ were plotted as a function of $(T/\eta)^{0.74}$ for the proteins labeled with SLI or of $(T/\eta)^{0.82}$ for the proteins labeled with SLII. From a least squares fit of the experimental data, we found $2\bar{A}$ and $(2\bar{A} - 2A')$ where $2A'$ corresponded to 20°C and 0% sucrose. The τ values of macromolecules were determined using the nomogram from [7] and the found value of $(2\bar{A} - 2A')$.

3. Results

3.1. *ESR spectra of spin-labeled proteins*

Figure 1 shows the ESR spectra of human IgG-SLI and Fc-SLI. At 20°C and 0% sucrose (fig.1, top

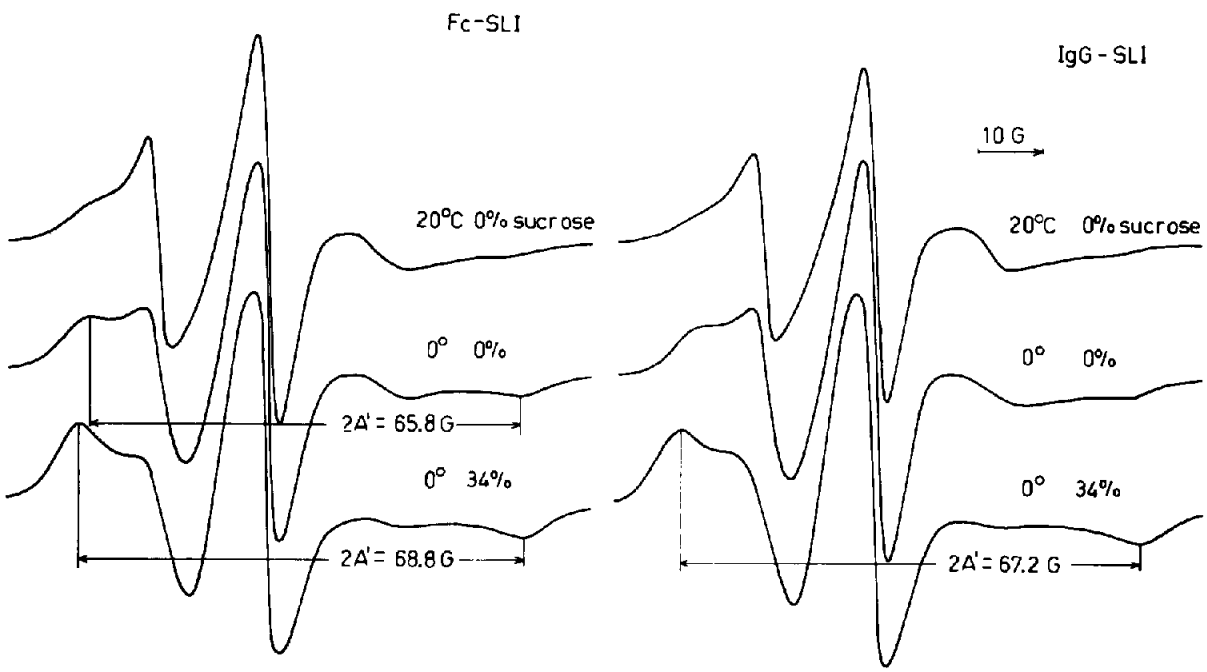

Fig.1. ESR spectra of human IgG and Fc spin-labeled at carbohydrates with SLI: top row, 20°C and 0% sucrose; second row, 0°C and 0% sucrose; bottom row, 0°C and 34% sucrose.

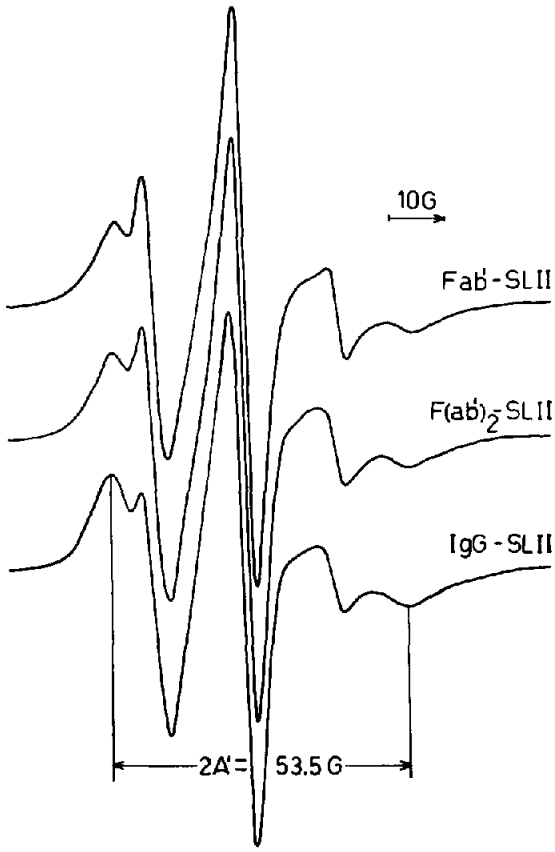

Fig.2. ESR spectra of rabbit IgG and its $F(ab')_2$ and Fab' fragments spin-labeled with SLII. 20°C and 0% sucrose.

row) ESR spectra reflect moderately immobilized rotation of SLI. The difference between ESR spectra in fig.1 and ESR spectra of IgG-SLI previously published [8] is due to the more complete removal of free spin-label in the present study. Decrease of temperature to 0°C results in the additional immobilization of SLI (fig.1, second row) and the appearance of well resolved outer extrema. The change of the distance between the outer extrema after addition of sucrose is demonstrated in fig.1, bottom row.

Figure 2 shows ESR spectra of rabbit IgG and its active fragments labeled with SLII. These spectra are similar to the previously described ESR spectra of immunoglobulins labeled with SLII [1] and have well resolved wide outer extrema even at 20°C and 0% sucrose. In this respect they differed from ESR spectra of proteins spin-labeled at carbohydrates (fig.1).

3.2. *Quantitative analysis of ESR spectra*

Figure 3 shows the dependence of $2A'$ on $(T/\eta)^{0.74}$ for Fc-SLI and IgG-SLI and on $(T/\eta)^{0.82}$ for rabbit IgG-SLII and its labeled fragments. Parameters necessary for the calculation of τ were obtained using these plots (table 1).

For IgG-SLI and Fc-SLI the $2\bar{A}$ (70 G) are almost equal to $2A_z$ (71 G) and this indicates the strong immobilization of SLI relative to the protein moiety. On the contrary, the $2\bar{A}$ values for IgG-SLII, $F(ab')_2$-SLII and Fab'-SLII are considerably lower (56.5 G), and this points to pronounced mobility of SLII relative to the protein moiety. The increase of viscosity by addition of sucrose above 45% produces a sharp rise of the $2A'$ values due to the strong immobilization of the relative spin-label rotation ($2A' \longrightarrow 2A_z$).

The values of τ obtained for IgG-SLII, $F(ab')_2$-SLII and Fab'-SLII correspond well to the values of τ

Table 1
Parameters of ESR spectra and rotational relaxation times (τ) of spin-labeled proteins

Spin-labeled proteins	$2\bar{A}$ (G)	$(2A_z - 2\bar{A})$ (G)	$(2\bar{A} - 2A')^a$ (G)	τ (ns)	Molar ratio spin-label/ protein
Human IgG-SLI	68.8	2.2	8.1	11	1.8
Human Fc-SLI	70.3	0.7	7.4	12	2.2
Rabbit IgG-SLII	56.5	14.5	3.9	26	3.9
Rabbit $F(ab')_2$-SLII	56.5	14.5	4.0	25	3.6
Rabbit Fab'-SLII	56.5	14.4	4.7	21	1.9

[a] $2A'$ was measured using the plots in fig.3 at (T/η)=300 (corresponding to 20°C and 0% sucrose concentration)

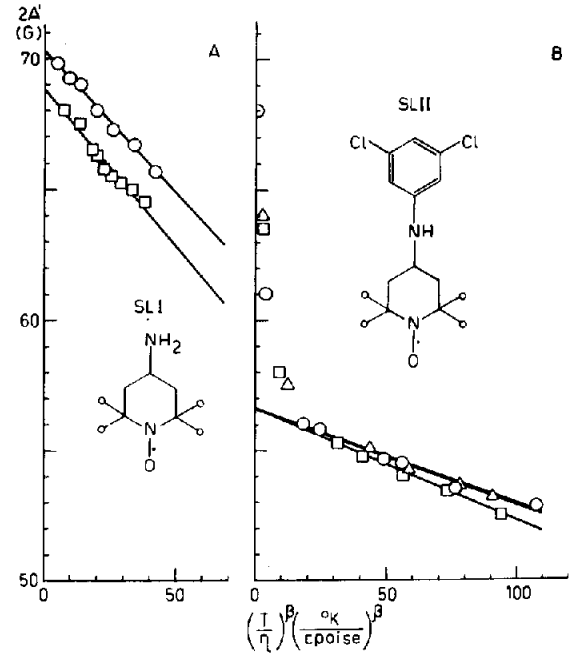

Fig.3. The distance between outer wide extrema ($2A'$) of ESR spectra versus $(T/\eta)^{\beta}$ for: A, human IgG-SLI (- □ -) and human Fc-SLI (- ○ -), $\beta = 0.74$; B, rabbit IgG-SLI (- ○ -), rabbit $F(ab')_2$-SLII (-△-) and rabbit Fab'-SLII (-□-), $\beta = 0.82$.

calculated previously by means of other methods for rigidly bound SLII in the combining site of anti-SLII or as in the present study for SLII bound non-rigidly [1]. These τ values (26 ns, 25 ns and 21 ns) are in a good agreement with the τ values calculated for Fab with mol. wt 50 000 [11], but the values of τ for IgG and Fc labeled at carbohydrates with SLI are about two times less (11–12 ns, table 1).

4. Discussion

The τ found for IgG-SLII, $F(ab')_2$-SLII and Fab'-SLII are approximately equal, and this points to the existence of segmental flexibility of IgG molecules. The observed value of τ for Fc-SLI is 2 times less than the value predicted for a rigid sphere of the same mass (mol. wt 50 000). This fact suggests the existence of an internal lability of Fc which seems to be necessary for its biological activity. All values of τ determined in this study for IgG as well as for the fragments correspond well to values obtained by fluorescence depolarization measurements [11–14].

According to X-ray data [15] Fc fragment is less compact than Fab fragment. The low value of τ obtained in the present study for Fc substantiates the existence of the loose structure of this fragment. The Fc oligosaccharide is closely attached to the C face of the C_H2 domain [15]. Our results prove the strong fixation of the oligosaccharide to the protein subunit of Fc relaxing with $\tau = 12$ ns.

In a previous study [8] on the basis of the shape of ESR spectra of IgG-SLI we concluded that SLI has marked freedom of rotation. But if the ESR spectrum is moderately immobilized as in our case, it is difficult to decide whether the shape of the spectrum is due to the significant freedom of rotation of spin-label relative to protein moiety or due to the rotation of spin-label rigidly bound to a protein subunit relaxing with $\tau \sim 10$ ns [4]. As we show above the shape of the ESR spectra of Fc-SLI is determined by such a low value of $\tau = 12$ ns. Besides, even a small admixture of unbound spin-label strongly affects the shape of such ESR spectra. In this study we used exhaustive dialysis to remove trace amounts of non-covalently bound spin-label.

Acknowledgement

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