SPIN–SPIN INTERACTION BETWEEN IMINOXYL RADICALS LOCALISED IN ANTIBODY COMBINING SITES*

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1. Introduction

It has been shown by using hybrid antibodies with the donor in one of the combining sites and the acceptor of fluorescent light in the other that the mean distance between the combining sites localised in Fab fragments of an intact IgG molecule exceeds 90 Å in solution at pH 7.5 [1]. This means that the relative Brownian rotation of Fab subunits is in some way limited despite the flexibility of the IgG molecule [2–4] and they are not able to come into contact with each other, at least in the area of the combining sites.

Previous experiments on the flexibility of IgG using fluorescence polarization [3] failed to reveal these limitations owing to the short life time of the excited state (7 nsec) of the dye used (dansyl group). This value only made it possible to estimate that the relative rotation of subunits was free over at least 25°. The nanosecond fluorescence polarization technique showed this rotation to be free over 33° [4].

The above results did not however explain the causes restricting the rotational freedom of IgG subunits and hindering, say, the 'sticking together' of Fab subunits. We now are attempting to throw some light on this question by studying the behaviour of antibodies against the spin-label: 2,2,6,6-tetramethylpiperidine-4-amino(*N*-dichlorotriazine) used as a hapten. Our study takes in consideration the possibility of estimating, in principle, the distance between the antibody combining sites in terms of the dipole–dipole interaction between the spins of the electrons of the iminoxyl radicals localised in the combining sites. The ESR method was previously used to investigate combining sites of anti-2,4-dinitrophenyl antibodies [5], anti-dansyl antibodies [6] and those with charged haptens [7]. To this end preparations of respective haptens with iminoxyl radicals were used which did not permit the complex of spin-label with active Fab fragments to be considered as absolutely rigid in contrast to our case when the hapten itself is a spin-label. The rigidity of specific complexes of spin-label with active Fab allowed the correlation times of such complexes to be determined.

2. Materials and methods

Rabbits were immunised two or three times with spin-labeled hemocyanin (Calbiochem) in complete Freund's adjuvant and IgG was chromatographically isolated from immune sera on DEAE-cellulose (DE 32, Whatman) in 0.0175 M phosphate buffer, pH 6.3. The content of antihapten antibodies in IgG preparations was equal to 3–5%. The specific antibody complexes with label were obtained by mixing 10% solution of IgG fraction in the above buffer with the spin-label solution. Specific complexes of the spin-label with papain and pepsin active fragments in hydrolysates resulting from the digestion of the IgG fraction with these enzymes (1% of IgG protein) were prepared in the the similar way. The change in the pH of solutions was achieved by adding 0.005 ml of the respective 2 M buffer after addition of 0.005 ml of the label in water to 0.1 ml of protein solution (0.02 M phosphate buffer, pH 6.3).

* *Abbreviations*: Ab = intact IgG antibody; R = spin-hapten; τ = the correlation time (The term ρ_h common to fluorescence polarisation for the rotational relaxation time is correlated with this term by $\tau = 1/3\ \rho_h$).

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ESR spectra were registered on an ESR-2-IChPh radiospectrometer provided with a thermostatted resonator cell.

3. Results and discussion

Mixing the IgG solution or its pepsin and papain hydrolysates with the label led to a drastic decrease in the mobility of the label (fig. 1). The presence of sharp components in the ESR spectra points to some excess label that failed to interact with combining sites.

The correlation times of specific complexes with spin-hapten were calculated from the shift of +1 component of ERS spectra according to McCally et al. [8] and were found to be 32 nsec, 31 nsec and 18 nsec for complexes of intact antibody, pepsin $F(ab')_2$ and papain Fab, respectively.

The shorter correlation time of isolated Fab points to a restricted freedom of rotation of Fab in the intact antibody molecule or in $F(ab')_2$.

It will be seen from fig. 1 that the central band of the ESR spectrum of the $F(ab')_2$ + R complex is abnormally wide (H_{max} = 11.7 G) as compared to spectral bandwidths of complexes Ab + R (H_{max} = 9 G) and Fab + R (H_{max} = 8.6 G). It is most likely that the additional widening of the band results from the dipole–dipole interaction between the spins of the electrons of the iminoxyl radicals localised in the combining sites. The averaging of the dipole–dipole interaction due to the rotation of the complex might remain unaccounted for, because the frequency of its rotation ($1/\tau \sim 10^8$ Hz) is many times lower than that of resonance transfers (10^{10} Hz) [9].

Table 1
Mean distance between the N–O hapten groups located in the combining sites of $F(ab')_2$ fragments of antibodies.

	25°		30°		40°	
	H_{max} (G)	r (Å)	H_{max} (G)	r (Å)	H_{max} (G)	r (Å)
Fab + R	8.6	–	8.3	–	7.6	–
Ab + R	9.0	–	8.5	–	7.7	–
$F(ab')_2$ + R	11.7	15.2	10.4	16.4	8.9	18.3

By additional widening of the central band of the ESR spectrum:

$$\delta(\Delta H)_{max} = [H_{max}(F(ab')_2 + R) - \Delta H_{max}(Ab+R)]$$

and introducing the expression for the second moment:

$$[\delta(\Delta H)]^2 = 3/5\ g^2\beta^2 s(s+1) \sum_k r_{jk}^{-6}$$

where $\delta(\Delta H)$ is $= \frac{\sqrt{2}}{2}(\Delta H_{max})$ if the band has a Gaussian curvature, β is the Bohr magneton and s is electron spin, it is possible to calculate the distance between two interacting spins:

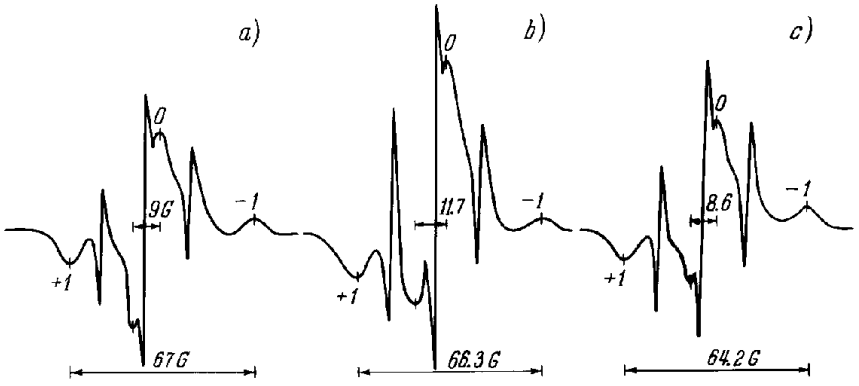

Fig. 1. ESR spectra of the spin-hapten mixed with solutions of: a) immune IgG; b) pepsin hydrolysate of immune IgG-($F(ab')_2$ fragments); c) papain hydrolysates of immune IgG (Fab fragments). The spectral components denoted by (+1, 0 and −1), respectively, correspond to three different orientations of the nitrogen nucleus spin in respect to the external magnetic field (0.1 M phosphate buffer, pH 6.3, 25°).

$$r = \sqrt[6]{\frac{155}{\delta(\Delta H)}} \cdot 10(\text{Å})$$

For $F(ab')_2$ + R at pH 6.3 and 25° this distance is found to be 15.2 Å (table 1).

Such spin–spin interaction may result from the drawing together of active sites of two Fab′ from the same $F(ab')_2$ molecule or from different $F(ab')_2$ molecules. The latter possibility is however unlikely because the $F(ab')_2$ with anti-hapten activity accounts for 3–5% of the total amount of $F(ab')_2$ molecules in our preparations. Furthermore the rotational correlation times of aggregates formed by two different $F(ab')_2$ is higher than the values of 31 nsec in our experiments for the $F(ab')_2$ + R complexes.

Hence splitting off of the Fc fragment leads to the 'sticking together' of the Fab′ fragments of the $F(ab')_2$ + R complex at this particular pH. The absence of a dipole–dipole band widening in the case of Ab + R is in good accord with the above-mentioned results that the distance between active antibody sites in the complex is not below 90 Å [1]. The possibility of changing interaction between IgG subunits as due to specific complex formation should also be borne in mind, for, as we showed [10] this is accompanied by conformational change in Fab fragments. Increased temperature of the solution of $F(ab')_2$ + R gives rise to longer mean distances between the combining sites (table 1).

It is to be noted that the dipole–dipole widening of the central component of the ESR complex of $F(ab')_2$ + R is observed only near the isoelectric point of Fab fragments (pH 6.3). No widening being observed over the pH ranges 4.5–5.5 and 7.5–8.9. Thus, in the region of the isoelectric point, the Fc fragment is the factor limiting the freedom of the movement of Fab subunits and stabilizing the Y-shape of the IgG molecule. When pH is further away from the isoelectric point the 'sticking together' of Fab′ fragments appears to be hindered by electrostatic repulsion. The distances between the combining sites in the $F(ab')_2$ + R at pH 6.3 are comparable to the Stokes radius (15–20 Å) of the domains of immunoglobulin peptide chains [11, 12]. This suggests that the combining sites are predominantly localised in one of the two domains (V_H and V_L).

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References

[1] Werner, T.C., Bunting, J.R. and Cauthou, R.E. (1972) Proc. Natl. Acad. Sci. U.S. 69, 795.
[2] Valentine, R.S. and Green, N.M. (1967) J. Mol. Biol. 27, 615.
[3] Zagyansky, Yu.A., Nezlin, R.S. and Tumerman, L.A. (1969) Immunochemistry 6, 787.
[4] Yguerabide, J., Epstein, H.F. and Stryer, L. (1970) J. Mol. Biol. 51, 573.
[5] Hsia, J.C. and Piette, L.H. (1969) Arch. Biochem. Biophys. 129, 296.
[6] Hsia, J.C. and Piette, L.H. (1969) Arch. Biochem. Biophys. 132, 466.
[7] Piette, L.H., Kiefer, E.F., Grossberg, A.L. and Pressman, D. (1972) Immunochemistry 9, 17.
[8] McCalley, R.C., Shimstick, E.J. and McConnell, H.M. (1972) Chem. Phys. Letters 3, 115.
[9] Slichter, C.P. (1963) Principles of Magnetic Resonance, Harper and Row, New York.
[10] Käiväräinen, A.I., Nezlin, R.S., Likhtenstein, G.I., Misharin, A.N. and Volkenstein, M.V. (1973) Molekul. Biol., in press.
[11] Karlsson, F.A., Peterson, P.A. and Berggard, I. (1969) Proc. Natl. Acad. Sci. U.S. 64, 1257.
[12] Poljak, R.J., Amzel, L.M., Avey, H.P., Becka, L.N. and Nisonoff, A. (1972) Nature New Biol. 235, 137.