EVIDENCE FOR MOBILITY OF IMMUNOGLOBULIN DOMAINS OBTAINED BY SPIN-LABEL METHOD

A.I. KÄIVÄRÄINEN and R.S. NEZLIN

Institute of Molecular Biology, Moscow and Institute of Biology, Petrozavodsk. The USSR Academy of Sciences, USSR

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SUMMARY: It is found that EPR spectra of immunoglobulins and their subunits spin-labeled by iminoxyl radical 2,2,6,6-tetramethyl-4-amino (N-dichlorotriazine) at pH 7.5 are similar in form and reflect the capability of spin-label to be in two states. Formation of specific complexes of spin-labeled antibodies with antigens is accompanied by increased correlation time of labels as well as by increased fraction of the labels in a more immobilized state. It is shown that splitting spin-labeled light chains to halves results in the label losing its capacity of being in the more rigid microenvironment. EPR spectra are interpreted as due to the relative motion of domains.

INTRODUCTION: Immunoglobulin peptide chains are constructed from relatively independent globulae stabilized by intrachain disulphide bridges and connected by less compact polypeptide stretches (1). This is substantiated by the possible proteolytic splitting of light chains to halves which retain their spatial structure as first proved by physico-chemical (2) and immunochemical studies (3), and finally by X-ray evidence (4, 5).

There are indications that antibody-antigen interaction may lead to conformational changes in antibody molecules (6,7). This work concerns results showing that these changes may be due to the capacity of globulae-domains for relative motion.

METHODS: Myeloma IgG was isolated on DEAE-cellulose column. IgE (ND) was kindly supplied by Prof. H. Bennich. Normal rat and human IgG were reduced by dithiotreitol (0.01 M) in 0.1 M Tris-HCl buffer, pH 8.0 and light chains were isolated by gel-filtration on a Sephadex G-100 column in 1 N acetic acid. Both type of light chains formed dimers in neutral solutions. Proteolysis

of light chains was carried out for 1.5 hr at 37° with 1 : 100 trypsin to protein ratio (3). Light chain halves were separated by gel-filtration on Sephadex G-50 (8). In experiments on the interaction of spin-labeled antibodies with antigens use was made of pure rabbit antibodies against human hemoglobin or bovine IgG and donkey antibodies against human IgG. Isolation of antibodies was performed by adsorption on corresponding antigens fixed on cellulose with subsequent elution by glycine-HCl buffer, pH 2.2. The specific complexes were formed at antigen-antibody ratios equal to 2 : 1 and only soluble complexes were analyzed. Proteins were labeled with two iminoxyl radicals: 2,2,6,6-tetramethyl-4-amino(N-dichlorotriazine) - R_1 and N - (2,2,5,5-tetramethyl-2,5-dihydropyrrolyl-carbonyl) imidazole - R_2. This was done by three different procedures. 1. Labeling with R_1 after treating protein with trichloro-S-triazin at pH 7.5 (0.01 M Tris-HCl). 2. Labeling with R_1 at pH 7.5 (0.01 M Tris-HCl). 3. Labeling with R_2 at pH 7.5 (0.01 M Tris-HCl). To the 1-2% protein solution was added a 10-20 fold molar excess of label dissolved in a small amount of dioxan. The mixture was stirred for 5-6 hr at room temperature, centrifuged and dialysed. The amount of spin-labels bound to a protein molecule was estimated by comparing the EPR spectrum of the spin-labeled protein with that of the reference spin-label solution at -140°C. EPR spectra were registered on radiospectrometer JES-ID-2.

RESULTS AND DISCUSSION: The first series of experiments involved EPR spectral data on human, rabbit and rat IgG as well as light chain dimers labeled with R_1 and R_2 by procedures 1 and 3. In these cases the EPR spectrum revealed three narrow components (Fig. 1), that is characteristic of rapidly rotating

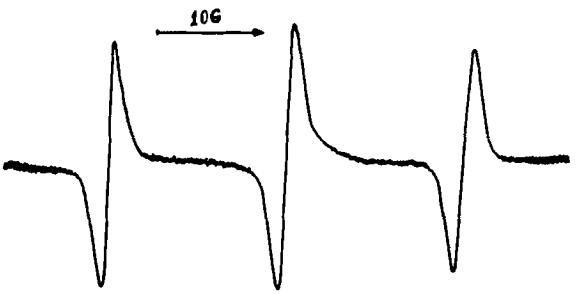

Figure 1. EPR spectrum of dimers of rat light chain spin-labeled by method 1. Protein concentration 10 mg/ml (0.01 M Tris-HCl, pH 7.5, 25°C). Label-protein molar ratio 3:1.

labels not sterically hindered by the macromolecule. The residues reacted with these labels appeared to be located on the surface of the molecule and were more likely lysine residues (9, 10).

On the other hand EPR spectral data on proteins labeled with R_1 at pH 7.5 by procedure 2, allowing the label to be bound to hystidine residues (9) revealed five components (Fig. 2). Immobilization of label (increased correlation time) led to the widening of EPR bands and the shift of side bands from the central one whose position remained relatively unaffected (11). If labels bound to proteins could remain in different states with different correlation time, the central bands of their spectra would overlap and the side bands would have different positions (A and B) on the tension axis of the magnetic field. This seemed to hold good in the case under study (Fig.2). These spectra accounted for IgG as well as for light chain dimers (Fig. 3) and $F(ab')_2$ and Fab fragments (7) with spectral shape not depending on the protein origin. There was a somewhat different relation of bands to be observed in spectra of spin-labeled IgE(ND) (Fig. 2) and IgE(Yu) previously reported (12) as compared with spectrum of IgG.

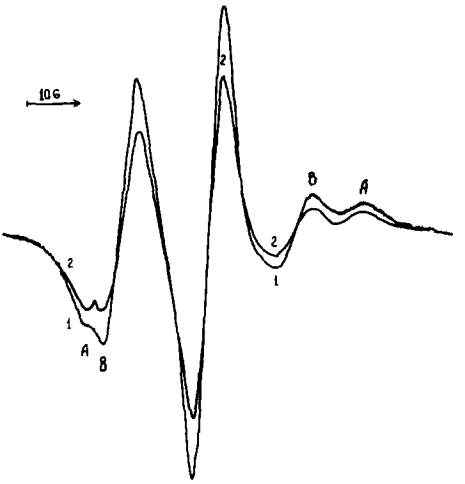

Figure 2. EPR spectra of human myeloma IgG (1) and IgE (2) spin-labeled by method 2. Protein concentration 15 mg/ml (0.01 M Tris-HCl, pH 7.5, 25°C). Label-protein molar ratio 2:1.

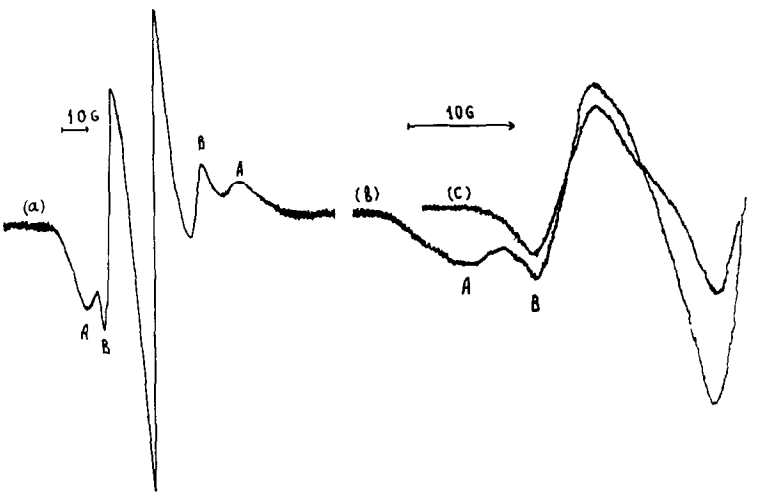

Figure 3. EPR spectrum of dimers of rat light chains spin-labeled by method 2 (a); left part of the same spectrum (b) and left part of EPR spectrum of halves of light chains spin-labeled by method 2 (c). Label-light chain dimers molar ratio 1:1. In experiments (a) and (b) η /T= $3 \cdot 10^{-5}$ P/deg (0.01 M Tris-HCl, pH 7.5, 25°C).

The resemblance of the form of EPR spectra for different spin-labeled immunoglobulins and their subunits might have been due to their common structural elements such as domains and

their interaction. This suggestion seemed to be indirectly substantiated by the splitting of labeled light chains to halves resulting in the complete disappearance of A components in EPR spectrum (Fig. 3). We assume that observed correlation time of spin-label depends not only on the motion of the label respectively the protein surface but also on rotation of spin-labeled macromolecule as a whole. Therefore, in this experiment viscosity of solution of light chain halves was increased fourfold by sucrose addition correspondingly to decreased molecular volume of light chain dimers after chain halving.

Interaction of spin-labeled antibodies with corresponding antigens (bovine IgG, hemoglobin) increased the area of A bands in respect to that of the central band as compared with the reference spectrum and resulted also in the shift of A components corresponding to increased immobilization of the label in A state (Fig. 4) (7). The area of A bands being determined by the

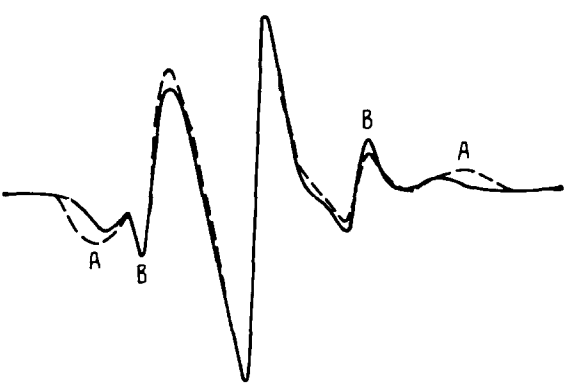

Figure 4. EPR spectrum of complex of hemoglobin and corresponding rabbit antibodies spin-labeled by method 2 (dotted line) and EPR spectrum of mixture of hemoglobin and non-specific rabbit IgG spin-labeled by method 2 (solid line). (0.1 M veronal buffer, pH 7.5, 25°C). Label-protein molar ratio 2:1. The label bound mainly (about 90%) to Fab fragments of antibody molecules.

amount of labels in this state, its increase pointed to an increased amount of labels in A state.

The form of EPR spectra (Fig. 2,3) could be accounted for by several causes. Firstly, it could result from overlapping of two spectral types that corresponded to two different sites of label binding with two different microenvironment, or secondly, from the capability of every label to be in two different microenvironments. The second explanation presumed the capacity of light chain dimers and Fab fragments to change their general structure in the process of domain Brownian motion. Thus A bands of EPR spectrum corresponded to a more compact A conformer, and B bands corresponded to a less compact B conformer of these proteins. The life-time of conformers had to be $\geqslant 10^{-7}$ sec otherwise A and B bands would have been smeared out (13). Thirdly, EPR spectrum depended on the combination of the first two causes.

The first explanation was not considered as reasonable, for interaction of spin-labeled antibody with antigen led not only to increased immobilization of the label in A state but also to increased amount of label in this state. This experimental evidence could be interpreted by the conformer model and was in accordance with 2nd and 3rd explanations of EPR spectra. It was thereby sufficient to assume that interaction of active Fab fragment with antigen led to equilibrium shift between A and B confromers towards A conformer and made the latter more compact. Such an interpretation of changes in EPR spectra of spin-labeled antibodies was suggested by us previously (7).

Our data are in agreement with the results obtained recently by Givol et al. (14). These authors using circular polarization of luminiscence found conformational changes in antibody

fragments as a consequence of antigen binding.

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