SPIN-LABEL STUDY OF SEGMENTAL FLEXIBILITY OF ANTI-HAPTEN ANTIBODIES

Precipitating pig anti-Dnp antibody is more flexible than non-precipitating

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1. Introduction

Relative freedom of rotation of the Fab-parts (Fab-arms flexibility) is very important for functional activity of the antibody molecules [1]. For example, cleavage of the inter-heavy chain disulfide bonds that results in conversion of incomplete rabbit antibodies into direct hemagglutinins points to an important role of Fab-parts movement [2]. In [3] we found by a fluorescence polarization method that precipitating pig anti-Dnp antibodies, randomly labeled by dansyl-chloride, had a more flexible structure than non-precipitating antibodies with the same specificity. The segmental flexibility of macromolecules can be determined by the spin-label method also [4]. Recently, a variation of that method has been described that permits evaluation of rotation of a spin-label relative to a macromolecule [5]. Using this method, the rotational correlation times τ of immunoglobulin molecules and their fragments were determined [6] and found to be in fair agreement with the results of fluorescence polarization experiments.

In this study, we determined the τ values of the early and late pig anti-Dnp antibodies with precipitating and non-precipitating properties. The spin-label was found to be attached both covalently to the protein moiety outside the combining site and non-covalently to the antibody combining site. Both these forms of label yielded identical rotational correlation times, showing that the precipitating anti-Dnp antibody is more flexible than the non-precipitating antibody.

Abbreviations: Dnp, 2,4-dinitrophenyl; Fab, antigen-binding moiety of antibody

2. Materials and methods

Pig anti-Dnp antibodies of the immunoglobulin G class were isolated as in [7] ans labeled with spin-label SL I (2,2,6,6-tetramethyl-*N*l-oxylpiperidine-4-amino-(*N*-dichlorotriazine) in 0.05 M phosphate buffer (pH 7.3) for 3 days at 4°C, or by spin label SL II (2,2,6,6-tetramethyl-*N*l-oxylpiperidine-4-iodoacetamide) in 0.1 M phosphate buffer (pH 8.0) for 1 week at 4°C. To remove the excess spin-label, exhaustive dialysis and gel filtration on Sephadex G-25 were used. The rotational correlation times τ were determined as in [5,6]. The ESR spectra of spin-labeled proteins were run at 20°C, and increasing sucrose concentrations ($\leqslant$40%) on a E-104 A Varian spectrometer. ϵ-Dnp–L-lysine (BDH, England) was used as a hapten.

Spin-label SL I was synthesized as in [8] with some modifications. Trichlorotriazine (275 mg, 1.47 mmol) and 4-amino-2,2,6,6-tetramethylpiperidino-oxy (250 mg, 1.46 mmol) (Aldrich) were dissolved in 20 ml absolute ethanol and stirred for 3 days at room temperature in the presence of dry $NaHCO_3$. The filtrate was evaporated and extracted with ether. The extract was washed with 10% acetic acid and water, dried by Na_2SO_4 and evaporated. The yield was 290 mg (1.11 mmol, 76%). The final product (m.p. 200–201°C) was homogeneous as established by a thin-layer chromatography on silica-gel plates

(Silufol UV-254, Czechoslovakia) in various solvent systems.

3. Results and discussion

The ESR spectra of pig anti-Dnp antibodies spin-labeled by SL I are similar to the studied ESR spectra of SL I labeled immunoglobulins of other species. The only difference is the additional outer wide extrema (A_2) which is apparently due to a more rigid immobilization of a fraction of the SL I molecules (fig.1a). The A_2 extrema disappear upon addition of ϵ-Dnp–L-lysine, or upon a 100-fold dilution of the sample by buffer. Simultaneously, sharp peaks appear that are characteristic of a free SL I label in solution (fig.1c). Obviously the specific hapten with a higher affinity displaced SL I from the antibody combining sites. Thus, the A_2 extrema is interpreted as a result of non-covalent binding of SL I by the antibody combining sites.

Both the high-field extrema (A_1 and A_2 in the right part of ESR spectra) are well resolved, in contrast to the low-field extrema (the left part of the spectra). Therefore, the τ values were calculated from the shift of the high-field extrema $\Delta H(-1)$ after increasing the solution viscosity [5,6]. The calculations were performed using the plot of the τ values against the shifts of the high-field extrema [5]. The parameters β for A_1 and A_2 extrema were equal to 0.76 and 0.67, respectively [5,6].

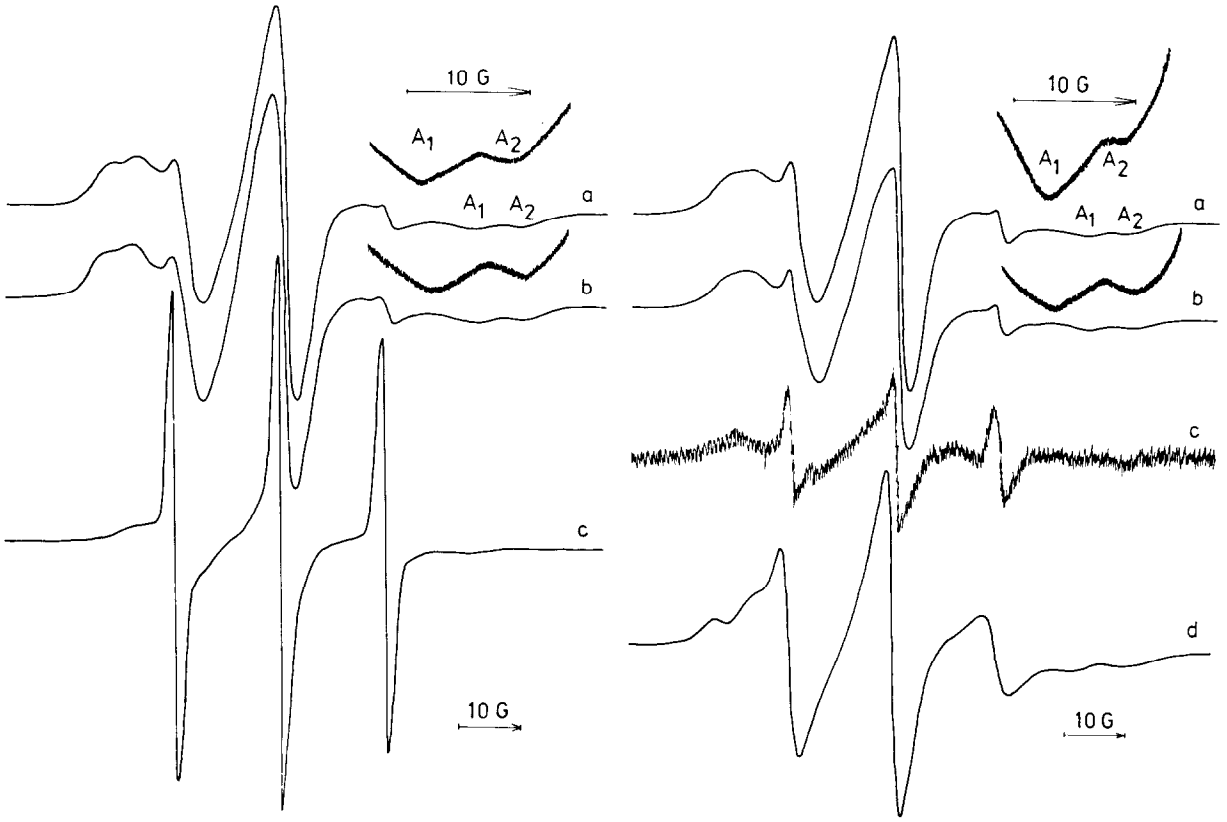

Fig.1. ESR spectra of spin-labeled pig anti-Dnp antibodies. Left: (a) SL I, non-precipitating antibody, 20°C, 0% sucrose; (b) SL I, non-precipitating antibody, 20°C, 20% sucrose; (c) SL I, non-precipitating antibody + ϵ-Dnp–L-lysine (molar ratio 1:20). Right: (a) SL I, precipitating antibody, 20°C, 0% sucrose; (b) SL I, precipitating antibody, 20°C, 20% sucrose; (c) SL I, non-precipitating antibody after 100-times dilution by phosphate buffer (pH 7.3); (d) SL II, non-precipitating antibody. High-field extrema are shown at higher gain at the right side of ESR spectra (a,b).

Figure 2 shows the dependence of $H'(-1)$ on $(T/\eta)^{\beta}$ for the precipitating and non-precipitating antibodies labeled by SL I. Parameters for calculation of the τ values were obtained using these plots (table 1). The rotational correlation times τ were very similar both for the label rigidly bound by the combining site (A_2) and for the covalently attached label (A_1). The τ values for the precipitating and non-precipitating antibodies were, however, markedly different. The higher value of the non-precipitating antibody suggests a higher rigidity of this antibody species.

The ESR spectra of the antibodies labeled by SL II appeared to be less useful since the outer wide extrema were poorly resolved (fig.1d).

The absolute values of the rotational correlation times obtained in this study and in the study employing fluorescence polarization [3] are different (19 and 29 ns versus 40 and 60 ns) in contrast to the similarity between the τ values for immunoglobulin G

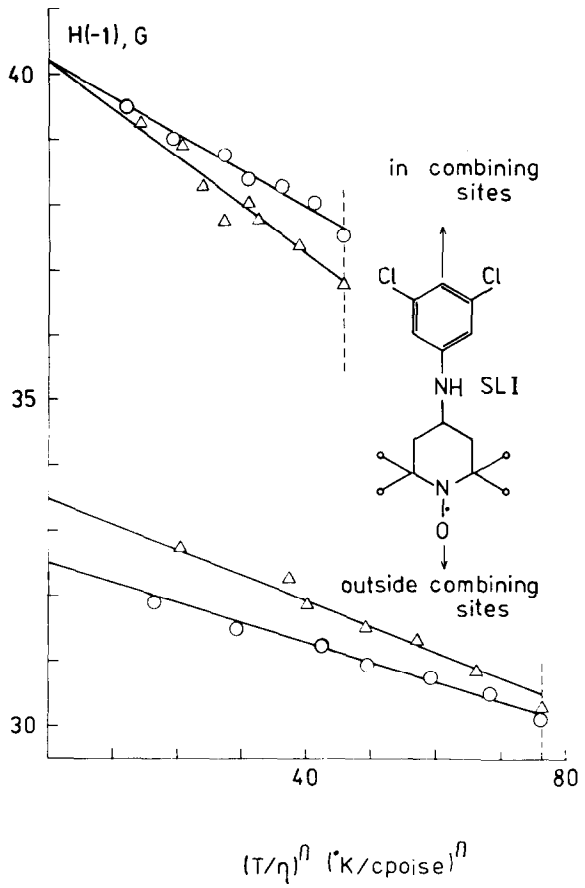

Fig.2. The distance of high-field extrema $H'(-1)$ from the central line of ESR spectra versus $(T/\eta)^{\beta}$ for the precipitating (△—△) and non-precipitating (○—○) SL I-labeled antibodies. Top: SL I localized rigidly in the combining sites ($\beta = 0.67$). Bottom: SL I localized non-rigidly outside the combining sites ($\beta = 0.76$).

Table 1
Rotational correlation times τ of pig anti-Dnp antibodies

Sample	Mode of SL I binding to antibody	$H'(-1)$ (G)	$\bar{H}(-1)$ (G)	$\Delta H(-1)$ (G)	τ (ns)
Precipitating antibody	Non-covalently, by combining sites	36.6	40.2	3.4	19 ± 1
	Covalently, outside combining sites	30.7	33.7	3.0	20 ± 1
Non-precipitating antibody	Non-covalently, by combining sites	37.6	40.2	2.6	29 ± 2
	Covalently, outside combining sites	30.1	32.5	2.1	28 ± 2

Definitions: $H'(-1)$, the distance of high-field A_1 or A_2 extrema from the central line determined at 20°C and 0% sucrose, i.e., $T/\eta = 300$ (see fig.2); $\bar{H}(-1)$, extrapolated value of $H'(-1)$ at $\eta \to \infty$, i.e., $T/\eta = 0$ (see fig.2); $\Delta H(-1) = \bar{H}(-1) - H'(-1)$, shifts of high-field extrema used for calculations of the τ values (see text)

determined by the two methods [6]. The reason for the discrepancy is not clear. The relation of the τ values obtained either by the spin-label method or by the fluorescence polarization method is, however, in good agreement with the view that the precipitating and the non-precipitating antibodies differ by their segmental flexibility.

Acknowledgement

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