FLUORESCENCE POLARIZATION ANALYSIS OF VARIOUS IMMUNOGLOBULINS

Dependence of rotational relaxation time on protein concentration and on ability to precipitate with antigen

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1. Introduction

Polarization fluorescence is widely used for the study of the general structure of immunoglobulin molecules. This method revealed that immunoglobulins possess segmental flexibility (Fab-arms flexibility) which varies between different classes and subclasses of these proteins and is very important for antibody functioning (reviewed [1,2]). We show in this paper that the rotational relaxation times (ρ_h) of immunoglobulins exhibit a significant dependence on immunoglobulin concentration. This effect is tentatively explained by loosening of the structure of the Fc part of the molecule in concentrations below 2 μM.

Taking into consideration the concentration dependence of ρ_h we performed a comparative polarization fluorescence study of two types of pig anti-DNP antibodies with different precipitation properties [3]. It was found that precipitating antibodies are more flexible than non-precipitating antibodies.

Abbreviations: ρ_h, rotational relaxation time; τ, the life-time of the excited state; DNS, 1-dimethylaminonaphtalene-5-sulfonyl; HGG and RGG, human and rabbit immunoglobulins G; HSA, human serum albumin; DNS–HGG, DNS–RGG, DNS–anti-DNP, DNS–HSA, DNS–Fab and DNS–Fe, DNS-conjugates of corresponding proteins; DNP, 2,4-dinitrophenyl

2. Materials and methods

HGG and RGG were isolated from commercial preparations of rabbit and human gamma-globulins by DEAE-chromatography. Rabbit peptic Fab′ fragments and human papain fragments were isolated from corresponding IgG as in [4,5]. Pig Fab was obtained after tryptic digestion of IgG. Pig antibodies (IgG class) were obtained as in [3].

Proteins were labeled by DNS-Cl (Calbiochem or Fluka). The number of DNS groups conjugated with a protein molecule was determined as in [6,7] and was found to be about 1–3 mol/mol. After DNS-labeling and subsequent dialysis protein solutions were gel filtrated through a K-26 column (Pharmacia) with Sephadex G-200 in 0.05 M Tris–HCl, pH 8, with 0.28 M NaCl for separation from protein aggregates.

The determination of the lifetime of the excited DNS-molecules (τ) and the degree of fluorescence polarization (p) as well as calculation of the rotational relaxation time (ρ_h) were performed as in [6–8]. The viscosity of protein solutions was changed by addition of concentrated sucrose solutions in such a way that the protein concentration was the same in all points of a given isotherm.

3. Results and discussion

3.1. *Concentration dependence of ρ_h for DNS–IgG and DNS–Fc*

The lifetimes of the excited state of DNS molecules and the Perrin-Weber isotherms were obtained for DNS–IgGs, their DNS-fragments, DNS–HSA and complexes of pig anti-DNS antibodies with DNS-lysine as a hapten (fig.1, table 1). Each isotherm corresponded to the change in viscosity on addition of sucrose at constant protein concentration. These curves for different concentrations of DNS–IgGs and DNS–Fc did not coincide with each other (fig.1A) in contrast to the curves for other investigated DNS-conjugates and complexes of anti-DNS with DNS-hapten (fig.1B). The values of ρ_h calculated from the isotherms for DNS–IgGs and DNS–Fc exhibited a marked dependence on protein concentration: in a narrow range of concentrations (around 2 μM) there was a sharp change of ρ_h (fig.2). This phenomenon was not observed with DNS–Fab, DNS–HSA and with complexes of anti-DNS antibody with DNS-lysine.

It seems likely that the concentration dependence of ρ_h for DNS–IgG and DNS–Fc is due to a partial

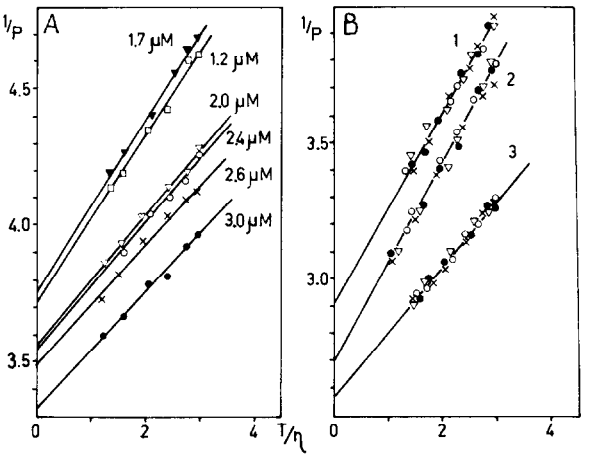

Fig.1. Dependence of reciprocal of the degree of fluorescence polarization ($1/p$) upon temperature divided by viscosity (T/η) for different concentrations of DNS–HGG (panel A) and pig DNS–Fab (curve 1), DNS–HSA (curve 2) and complexes of pig anti-DNS antibodies with DNS-lysine (curve 3) (panel B). In panel A the protein concentrations are shown on the curves; in panel B different symbols on the same curve refer to experimental points obtained at different protein concentrations: (○) 1.3 μM; (×) 1.6 μM; (△) 3 μM; (●) 4 μM. Wavelength of excitation by polarized light 365 nm, T/η in deg $P^{-1} \times 10^{-4}$, 20°C. Viscosity was changed by addition of concentrated sucrose solution.

Table 1
Rotation relaxation times (ρ_h) and lifetimes of the excited state (τ) for DNS–protein conjugates and complexes of anti-DNS antibodies with DNS-hapten

Protein	ρ_h min[a] (ns)	ρ_h max[a] (ns)	τ (ns)
Human DNS–IgG	67	90	7.2
Rabbit DNS–IgG	64	82	7.3
Precipitating pig DNS-antibodies to DNP group	83	123	8.6
Non-precipitating pig DNS-antibodies to DNP group	122	188	8.0
Complexes of pig anti-DNS antibodies with DNS-lysine	162	162	17.2
DNS–Fab from rabbit IgG	63	63	7.5
DNS–Fab from pig IgG	62	62	9.5
DNS–Fab from human IgG	65	65	7.4
DNS–Fc from human IgG	28	49	7.0
DNS–human serum albumin	105	105	17.7

[a] Mean of 5–8 determinations for several preparations. SD was less then 7%. ρ_h min corresponds to protein concentrations below 2 μM; ρ_h max, higher than 2 μM

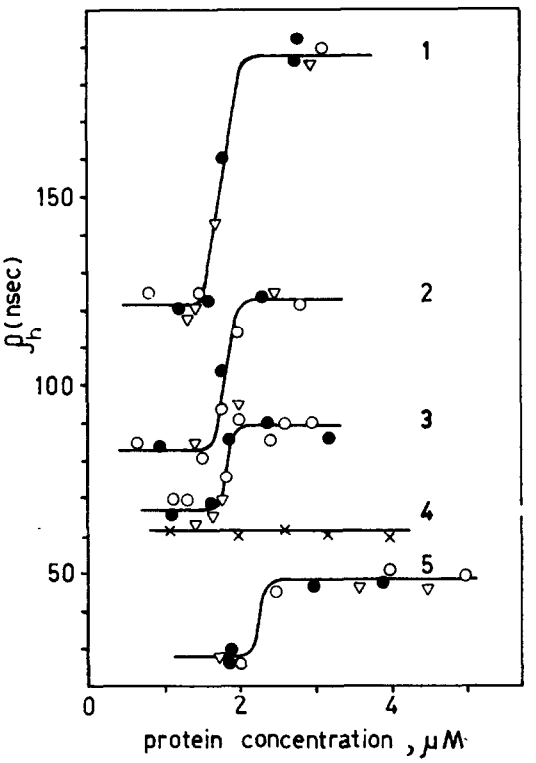

Fig.2. The dependence of the rotational relaxation time on protein concentration for DNS-conjugates of non-precipitating pig anti-DNP antibody (curve 1), precipitating pig anti-DNP antibody (curve 2), human IgG (curve 3), pig Fab (curve 4) and human Fc (curve 5). Different symbols on the same curve refer to experimental points obtained in individual experiments.

dissociation or loosening of Fc part of immunoglobulin molecules after dilution below 2 μM. Upon IgG labeling some DNS groups also bind to Fc parts and it is very probable that the partial dissociation of Fc into smaller subunits which rotate more freely, could result in the diminishing of ρ_h of the whole molecule. This assumption is substantiated by the fact that no concentration dependence of ρ_h was found for DNS–Fab or for the complexes of anti-DNS with DNS-lysine located in the combining sites of the Fabs. The steepness of the ρ_h change may indicate that the loosening of the structure depends on a cooperative dissociation of several non-covalent bonds.

After addition of unlabeled HGG to solutions with low concentration of DNS–HGG (1 μM) the measured ρ_h increased correspondingly to the whole protein concentration of the sample. But if unlabeled HSA was added to the same solution of DNS–HGG there was no change in the ρ_h value. The results of this control experiment are in a good agreement with the proposed hypothesis.

The dissociation of Fc perhaps depends on its loose structure previously known from its pronounced susceptibility to proteolysis and now proved well by X-ray crystallographic studies [9]. The measured ρ_h for Fc is lower than the ρ_h for the Fab (table 1) despite of the similar molecular weight of both fragments. This also points to a definite lability of the Fc structure.

3.2. *Comparison of ρ_h for precipitating and non-precipitating pig anti-DNP antibodies*

Taking into account the concentration dependence of ρ_h we determined the values of ρ_h for two types of pig IgG antibodies against the DNP hapten. The antibody isolated at an early phase of the immune response precipitated effectively DNP-substituted proteins [3]. Its ρ_h values were significantly lower than the values for the non-precipitating antibody isolated at a late phase of the immune response (table 1). We can conclude from these data that non-precipitating anti-DNP antibody had a less flexible structure, which could affect the ability to cross-link the molecules of DNP-antigens. The difference of the radii of gyration of the two antibody types [10] is compatible with this conclusion.

The Fab arms flexibility was shown several years ago by steady-state fluorescence polarization measurements of DNS-immunoglobulins, their fragments and specific complexes of anti-DNS antibody with DNS-hapten [6,7,11] as well as by the ns technique [12]. Recently these observations were confirmed by ns fluorescent spectroscopy measurements of specific antibody complexes with another fluorescence dye, pyrenylbutyrate [13].

It follows from the above and from [14] that the extent of Fab flexibility can seriously affect functional properties of antibodies, particularly the capacity to form specific precipitates with an antigen. Recent observations on conversibility of incomplete antibodies to direct agglutinators by mild reduction [15] also points to the important role of Fab movement on the character of antibody reaction with polyvalent antigens.

Acknowledgement

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