INCREASE OF THE ROTATIONAL RELAXATION TIME OF ANTIBODY MOLECULE AFTER COMPLEX FORMATION WITH DANSYL-HAPTEN

L.A. TUMERMAN, R.S. NEZLIN and Y.A. ZAGYANSKY
Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow V-312, USSR

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1. Introduction

In our previous reports [1, 2] we presented evidence on the flexibility of the bonds between subunits corresponding to the Fab and Fc fragments of immunoglobulin G. The problem now posed is whether this high structural flexibility of the antibody is retained when the antibody is complexed with its corresponding antigen. To elucidate this problem the fluorescence of dansyl (DNS) groups was studied after their specific combination with the combining site of anti-dansyl antibodies.

2. Materials and methods

Rats were immunised twice, with a month's interval, with 2 mg of the DNS conjugate of bovine gamma-globulin (BGG) in complete Freund's adjuvant. Two months later another 2 mg of DNS–BGG was injected subcutaneously in saline. Blood was taken for examination 8 days after this injection. Immunoglobulin G (IgG) was chromatographically isolated from immune and non-immune sera on DEAE-cellulose (Whatman DE-32) in 0.0175 M phosphate buffer, pH 6.3. The papain Fab fragments were isolated as previously described [2]. Peptic $F(ab')_2$ fragments were reduced to univalent fragments with 0.1 M dithiothreitol and alkylated with 0.11 M iodoacetamide. ε-DNS-lysine (Calbiochem) was used as a hapten. Control fluorescence measurements showed the hapten to be bonded only to the antibody against DNS and not to non-specific IgG.

The polarisation of fluorescence (P) was estimated by a device with a rotating analyzer, the duration of the excited state of the DNS molecules was measured with a phase fluorometer. These devices, as well as the methods of calculation of ρ_h were as already reported [1, 2].

3. Results and discussion

In table 1 are compared the values of the parameters of fluorescence: the duration of the excited state τ and the position of the fluorescence maximum λ^{fl}_{max} for the DNS conjugates with non-specific IgG and for complexes of DNS with anti-DNS antibodies. It will be seen that the fluorescence characteristics of the dansyl groups complexed with the combining site of the antibody are markedly different from those of the same groups that are conjugated non-specifically with IgG. The values of τ increase from 7.5 to 17.3 nsec and the maximum of spectral distribution shifts from 543 to 490 nm.

This difference in fluorescence involves yet another important difference in the properties of the complexes being compared. The polarization results are presented in terms of the dependence of $1/P$ on T/η. With non-specific conjugates a considerable difference is observed between the plots obtained on heating the solution and those obtained for the isothermal change in viscosity after addition of sucrose [1, 3, 4]. Yet with DNS–anti-DNS complexes these curves coincide (fig. 1). This means that the dansyl groups are fixed rigidly in the combining sites of the antibody and are thus devoid of that relative freedom they display in non-specific conjugates.

The mutual correlation between the above changes in the fluorescence parameters and the relation of

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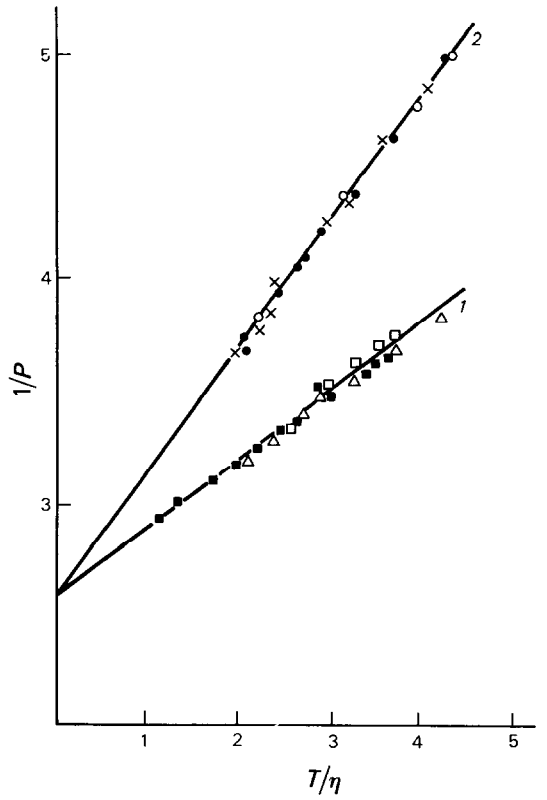

Fig. 1. Dependence of the reciprocal of the fluorescence polarisation ($1/P$) upon temperature divided by viscosity (T/η) for complexes of DNS with intact anti-DNS antibody (1) and with its Fab and Fab' fragments (2). The initial buffer was 0.28 M NaCl–0.1 M Tris-HCl, pH 8.0. Curves for intact antibodies obtained at 28° (■), on heating (△) and cooling (□); for papain Fab fragments on heating (x); for peptic Fab' fragments on heating (●) and cooling (○).

these differences to the rigid fixation of DNS molecules on proteins have also been reported for several other DNS–protein conjugates [1]. On the other hand, the fluorescence study of DNS complexes with lysine and some other amino acids in solution showed that increased hydrophobicity of the environment of dansyl groups leads to the same changes in fluorescence such as increased τ-values and fluorescence spectrum shift to the short wave side, as observed by us on the fixing of DNS in the antibody combining site [1, 5, 6]. These facts suggest the DNS molecules complexed with the antibody combining sites to be in a relatively hydrophobic environment.

Table 1 gives also ρ_h-values for non-specific DNS conjugates and DNS–anti-DNS complexes, calculated from Perren–Weber plots similar to those presented in fig. 1. It will be seen from table 1 that the values of ρ_h for Fab antibody fragments combined with hapten and the same fragments of non-specific IgG are almost the same. However, for the intact antibody molecules complexed with hapten the ρ_h -value is twice that for Fab fragments, whereas with non-specific DNS–IgG conjugates these values are very close to each other.

Such a difference might be explained by assuming that when hapten is bonded to the combining site, the conformation of the antibody molecule changes so that its subunits corresponding to the Fab and Fc fragments become more rigidly interlinked. This results in an increased contribution of the rotation of the antibody molecule as a whole to the observed depolarisation of fluorescence.

This assumption can be substantiated by semi-quantitative calculations based on experimental evidence reported by Yguerabide et al. [7] on the fluorescence of DNS–lysine complexes with antibodies against DNS and their Fab fragments. It was found by the pulse method that for Fab fragments the decay

Table 1
Parameters of the fluorescence and ρ_h-values of DNS–IgG conjugates and of complexes DNS–anti-DNS antibodies.

	τ (nsec)		λ^{fl}_{max} (nm)		ρ_h (nsec)	
	Antibody	Non-specific IgG	Antibody	Non-specific IgG	Antibody	Non-specific IgG
Intact molecule	17.3	7.5	490	543	110	60
Fab-fragment	–	7.9	–	–	64	66

The values of ρ_h were calculated at $T/\eta = 3.34 \times 10^4$ deg/poise.

curve of anisotropy $A(t)$ was an exponent with the constant ρ_1 = 99 nsec, whereas for the intact antibody it had a much more complicated form and could be approximated by the sum of two exponents.

$$A = A_0 [(1-\beta) \exp(-3t/\rho_1) + \beta \exp(-3t/\rho_2)] \qquad (I)$$

with ρ_1 = 99 nsec, ρ_2 = 504 nsec and β = 0.56, A_0 being the limiting emission anisotropy corresponding to the lack of Brownian rotation. The authors interpreted ρ_1 = 99 nsec as being the relaxation time of Fab fragments and ρ_2 = 504 nsec as the relaxation time of the whole molecule, with β corresponding to the relative contribution of the latter.

It can be easily shown that if the decay curve of anisotropy is represented by (I), the stationary values of fluorescence polarisation on permanent excitation are expressed by (II) which can be derived from, for example, equation (2) of Jabloński [8]:

$$\frac{1/P - 1/3}{1/P_0 - 1/3} = \frac{1}{\dfrac{1-\beta}{1+\dfrac{3\tau}{\rho_1}} + \dfrac{\beta}{1+\dfrac{3\tau}{\rho_2}}} \qquad (II)$$

It is now possible to calculate, using (II), the dependence of $1/P$ on T/η and to show that over the range of T/η studied, and with reasonable values of ρ_1, ρ_2, τ and β, the plot of this dependence is a good approximation to a straight line just as is the classical Perren–Weber plot. Calculating ρ_h conventionally from the slope and intercept of this plot gives, however, only an effective value, $\rho_{eff.}$, which depends both on the constants ρ_1, ρ_2 and β for the protein particle and on τ for the fluorescent label used.

With ρ_1, ρ_2 and τ given it is possible to calculate the dependence of the experimentally estimated $\rho_{eff.}$ on β for a particular value of T/η. Such calculations, made for ρ_1 = 60 nsec, ρ_2 = 300 nsec with 3τ = 20 nsec (non-specific conjugates) and 3τ = 50 nsec (DNS–anti-DNS complexes) showed that the value of β for DNS–IgG conjugates cannot exceed 0.1 and should actually be even lower. As to DNS–antibody complexes the experimental value of $\rho_{eff.}$ = 110 nsec corresponds to β = 0.42. This value of β is close to that derived quite differently [7]. This appears to confirm the validity of our assumption that the complexing of the antibody with hapten strongly decreases the flexibility of bonds between IgG subunits. It is yet not clear whether the phenomenon described is of a general nature or caused by the interaction of the two haptens complexed with the two combining sites of one antibody molecule. Should it be of a general nature it could have an important bearing on the elucidation of the biological functions of the antibody.

Flexibility is obviously extremely valuable at the recognition stage, as it enables the antibody to recognise the antigen and favours their complexing. The following decrease in flexibility does not, however, adversely affect recognition and the conformational changes leading to decreased flexibility might possibly enhance other biological functions of the antibody.

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