RESTRICTED FLEXIBILITY OF CARP 15 S IMMUNOGLOBULIN MOLECULES AS REVEALED BY FLUORESCENCE POLARISATION

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1. Introduction

Immunoglobulins of teleosts (bony fishes) are made up of heavy and light chains as are immunoglobulins of other vertebrates. For different species of teleosts, high molecular weight immunoglobulins, built up from four subunits linked by disulphide bridges and non-covalent bonds have been described [1,2]. These proteins of teleosts are similar to mammalian IgM (electrophoretic mobility of heavy chains, amino acid composition and carbohydrate content). There are, however, some differences, for example in precipitating properties. Antibodies of teleosts precipitate only in high antigen excess [3] and require for optimal precipitation a large number of repeated antigenic determinants on the carrier molecule [4]. One of the possible explanations of these functional peculiarities may be a restricted flexibility of the immunoglobulins of teleosts. The purpose of this study is the elucidation of this possibility by the fluorescence polarisation technique previously used for the investigation of IgG and IgM of mammals [5–8].

2. Materials and methods

The immunoglobulin was isolated from serum of carp (*Cyprinus carpio* L) by two different methods. The first preparation (Ig-I) was isolated by precipitation with octanoic acid (Acro, Berlin) (pH 4.5; 2.7%). The precipitate was dissolved in saline and dialysed against it. The second preparation (Ig-II) was isolated by sodium sulphate precipitation (20% w/v) and starch gel block electrophoresis in barbital buffer, pH 8.6 and $\mu = 0.1$ (400 V, 50–60 mA). Both preparations were gel-filtered through Sephadex G-200 and lyophilized. Only one line was noticed on immunoelectrophoresis and disc electrophoresis. Both proteins sedimented as one sharp peak in the analytical ultracentrifuge ($S^{\circ}_{20,w} = 15.0$ S). Ig-I and Ig-II were stained with 1-dimethylaminonaphtalene-5-sulfonyl (DNS) chloride (Fluka) and extensively dialysed against 0.28 M NaCl/0.05 M Tris-HCl, pH 8.0. The molar ratios of DNS to protein calculated from the absorbancy at 280 nm and 340 nm were 22.0 for DNS–Ig-I and 16.5 for DNS–Ig-II. The molecular weight of carp immunoglobulin was taken as 700 000 and the absorption coefficient as $A_{280}^{1\%}{}_{nm,\,1cm} = 12.0$. The molar extinction of the DNS group was taken from Weber [9]. Before fluorescence polarisation measurements both samples were gel-filtered through columns of Sepharose 6B (Pharmacia) equilibrated with 0.28 M NaCl/0.1 M Tris-HCl, pH 8.0. Both DNS–Ig-I and DNS–Ig-II separated into two peaks, the first of which (about 15%) contained aggregated immunoglobulin molecules. Only the fractions corresponding to the second peak were used in polarisation measurements.

The lifetime of excited DNS molecules (τ) was determined on the phase fluorometer, the degree of fluorescence polarisation (p) was measured by means of a device with a rotating analyser [5]. Fluorescence polarisation measurements were carried out at a con-

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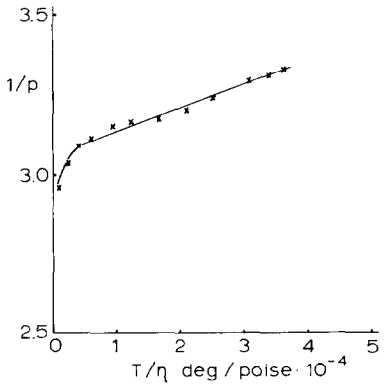

Fig. 1. Dependence of the reciprocal of the fluorescence polarisation ($1/p$) upon temperature divided by viscosity (T/η) for the DNS conjugates of carp Ig-I (25°). Wavelength of excitation by polarised light 365 nm.

stant temperature (25°) in solutions the viscosity of which was changed by adding various concentrations of sucrose. For the preparation of Tris buffer and sucrose solutions with low background fluorescence Aristar-grade reagents supplied by BDH (England) were found more suitable.

3. Results and discussion

The results were graphically presented as "Perrin-Weber" plots giving the $1/p$ values as a function of the ratio T/η (fig. 1). The values of ρ_h* were calculated from these plots using the formula

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p'_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right)$$

Here $1/p'_0$ is the value corresponding to the intercept of the extrapolated linear part of the plot with the $1/p$ axis. It was shown [11,13] that this way of calculating ρ_h practically eliminates the error arising from the possible thermally activated freedom of independent rotation of the dye molecule.

For the mean lifetime of excited DNS molecules conjugated with Ig-I and Ig-II we have found the values of τ = 11.5 nsec and 10.5 nsec respectively. With these τ values we obtained from experimental plots like that in fig. 1 the values of ρ_h = 390 nsec and ρ_h = 380 nsec.

In table 1 the experimental values of ρ_h for carp immunoglobulins as well as for human immunoglobulins G and M and for Fab fragments of IgG are compared with the theoretical values (ρ_0) calculated for rigid spherical protein particles of corresponding molecular weight. In these calculations the mean values 0.735 cm^3/g for protein specific density and −0.3– −0.37 g/g for its hydration were taken.

One can see from table 1 that for carp as well as for human immunoglobulins the theoretical values of ρ_0 are sometimes greater then the experimentally found values of ρ_h. This leads to the conclusion that carp immunoglobulins like those of mammals contain some smaller subunits interconnected by flexible linkages.

There is however the significant difference between carp and mammalian immunoglobulins: while for the latter the values of ρ_h of the intact molecules are very close to those of their Fab fragments, for carp immunoglobulin the value of ρ_h for the intact molecule is sometimes greater than that for Fab of human and rat IgG.

It was also shown [6,16] that the experimental ρ_h for Fab fragments are very close to those calculated for rigid particles with the same dimensions approximated as rotational ellipsoids with axial ratio 1:2 [14,15] ($\rho_h$calc. = 64 nsec). We have concluded that the linkages between the fragments of mammalian immunoglobulins are quite flexible and the Brownian rotation of each fragment is practically independent of the rotation of other fragments [6], at least during the time equal to the lifetime (τ) of the excited state of the conjugated dye molecule. However the possibility exists that there are some hindrances which do not allow the fragments to turn to greater angles. In the two extreme cases of completely rigid or of completely free linkages the values of ρ_h calculated from Perrin-Weber plots represent true constants characteristic in the first case for the whole molecule or in the second case for its fragments. In all intermediate cases we obtain from these plots only the "effective" values of ρ_h which depend on many factors including the degree of flexibility of linkages and the τ of the fluorescent label used [16]. Thus Knopp and Weber [17], who have found ρ_h = 1000 nsec for human IgM labeled with pyrenebutyrate (τ = 100 nsec), believe that there are no contradictions between

* ρ_h is the harmonic mean of the principal relaxation times of rotation of ellipsoidal molecule [11,12].

Table 1
Lifetime of the excited state and rotational relaxation time of DNS-conjugates of carp and mammalian immunoglobulins.

DNS conjugate	τ (nsec)	ρ_h exp (nsec)	Mol. weight	ρ_0 calculated (nsec)
Carp Ig-I	11.5	390	7×10^5	700
Carp Ig-II	10.5	380	7×10^5	700
Human IgM (Pr)*	8.5	140	9×10^5	1000
Human IgM (Sch)*	8.5	80	9×10^5	1000
Rat and human IgG**	6.2– 7.5	60– 62	15×10^4	160
Fab of human IgG**	7.9	64	5×10^4	53
Complex of dansyllysine with Fab of rat antidansyl antibody***	17.3	66	5×10^4	53

* Taken from [8].
** Taken from [6].
*** Taken from [16].
($T/\eta = 3.34 \times 10^4$ deg/poise, water, 25°).

their data and previous data of Metzger et al. [18] who found, for the same molecule labeled with DNS, the value of ρ_h = 60–80 nsec very close to the one found by us with the same label [8].

If subunits similar to the Fab fragments of mammalian immunoglobulins are present in carp immunoglobulin, one could conclude from our data that the linkages between them are much more rigid in the phylogenetically ancient carp immunoglobulin than in mammalian immunoglobulins. It seems likely that the evolution the structure of the antibody molecules has changed in the direction of greater flexibility, because this property favours the antigen–antibody recognition and interaction.

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