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Fluorescence assessment of antibody binding and molecular interactions

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Abstract

We observed a pronounced decrease in the binding affinity of TMR to the immunoglobulin specific for this dye upon adding β-cyclodextrin. Experimental evidence suggests that TMR interacts simultaneously with the IgG antigen binding site and with the CD cavity. Fluorescence anisotropy was employed to further characterize the nature of the interactions between TMR and IgG. It is found that TMR binds with high affinity to IgG, but retains its ability to rotate within the antigen binding site.

Keywords: Immunoglobuling affinity; Cyclodextrins

1. Introduction

Antibodies are glycoproteins elicited by the adaptative immune system that serve to eliminate noxious substances and microbials [1]. The investigation of the binding properties of small fluorescent molecules to antibodies has been important for the characterization of the interactions involved in the formation of antigen–antibody complexes. This research direction commenced with the classic study of Lopatin and Voss of the fluorescence properties of fluorescein using anti fluorescein antibody as a quencher [2]. Subsequently, this system attracted intense interest [3] and culminated with the determination of the X-ray structure of several antigen–antibody complexes [4]. Shreder et al. studied the binding properties of monoclonal antibodies specific for a family of ruthenium complexes, and were able to probe subtle structural changes because the fluorescence lifetimes of these complexes are long and their fluorescence yields are sensitive to changes in the microenvironment [5]. Iwaki et al. took a different approach to investigate hapten-antibody active site interactions [6]. The notion that the formation of the antigen–antibody complex inhibits the rotation of 9-(2-carboxy 2-cyanovinyl)julolidine (CCVJ), thereby increasing its fluorescence quantum efficiency, was employed by these workers to obtain information about the rigidity of the CCVJ-antibody complex. More related to our work, Yguerabide et al. examined the time-resolved polarized fluorescence of dansyl bound to the anti dansyl antibody and demonstrated that the fluorescence anisotropy decays consist of two component that they assigned to the brownian rotational motion of the whole IgG molecule and to a local motion of the F_{ab} moiety [7].

Surprisingly little is known about the effects that foreign molecules may have on the antigen–antibody binding affinity. In this paper, we would like to shed some light on this problem studying the influence of β-cyclodextrin (CD) on the binding affinity of TMR (antigen) to anti TMR antibody. We selected CD as an example because this molecule has a cavity to which TMR can bind partially and the equilibrium, TMR + CD ↔ TMR-CD, can be studied separately [8]. Furthermore, CDs are transparent in the region of the spectrum where TMR and IgG absorb light. Our main finding is that the binding affinity of the antigen–antibody complex decreases steadily up to a fifth of the original value upon adding CD. A second aspect of our work was to assess how rigidly TMR binds to IgG [9]. TMR has the remarkable features that it can emit when it is bound to IgG and its position is exactly known. We measured a large anisotropy for the TMR-IgG complex, which gives a complementary method to characterize the binding process [10].

Abbreviations: TMR, tetramethylrodamine; CD, β-cyclodextrin; IgG, immunoglobulin; CCVJ, 9-(2-carboxy 2-cyanovinyl)julolidine; F_{ab}, fragment binding antigen; F_c, fragment crystallizable

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2. Experimental

2.1. Chemicals

Tetramethylrhodamine (5′-carboxylic isomer), polyclonal anti-tetramethylrhodamine rabbit antibody (1 mg/1 mL, in phosphate-buffered saline, pH 7.2, containing 5 mM sodium azide), were purchased from Molecular Probes, whereas β-cyclodextrin, HCl, Na_2HPO_4, $NaH_2PO_4{\cdot}2H_2O$ from Aldrich. All solutions were prepared using fresh distilled-deionized water and the pH was adjusted to 7.2 using the phosphate buffer, as described elsewhere [11].

2.2. Absorption and fluorescence measurements

The concentrations of TMR solutions were measured monitoring its absorption band ($\varepsilon = 7\times 10^4\,M^{-1}\,cm^{-1}$ at 552 nm). UV–vis spectra were recorded at room temperature using a Varian Cary 50 spectrophotometer. Steady-state fluorescence measurements as well as anisotropy measurements were performed in a Varian Cary-Eclipse spectrofluorometer. A magnetic stirrer was adapted to the sample cell to ensure continuous mixing of the solutions. The temperature of the solutions was controlled using a water-cooled Single-Cell-Peltier-Accessory (Varian).

2.3. Fluorescence quenching assay

The reduction in the fluorescence quantum yield of TMR when it binds the antibody, was employed to construct Scatchard plots [12,13]:

$$\frac{r}{c} = K_a n - K_a r \tag{1}$$

where K_a is the association constant, n the valence of the binding protein ($n = 2$ for IgG), and r is the moles of dye bound per mole of anti-dye antibody defined as

$$r = \frac{[TMR]_b}{[IgG]} = \frac{[TMR]_0 - [TMR]}{[IgG]} \tag{2}$$

where $[TMR]_b$, $[TMR]$ and $[TMR]_0$ refer to the total concentration of bound dye, free dye and total dye present in solution, and c is the molar concentration of free ligand at the end of each titration. r and c were retrieved from our fluorescence data. The observed fluorescence intensity (F) (per mole of dye) resulted from two contributions,

$$F = F_0 + F_b \tag{3}$$

where F_0 and F_b are the mole fluorescence intensities for free and bound dye. In the Scatchard equation the parameters r and c are given by:

$$r = [TMR]_0 \frac{F - F_b}{F_0 - F_b} \tag{4a}$$

$$c = \frac{[TMR]_0}{[IgG]} \frac{F_0 - F}{F_0 - F_b} \tag{4b}$$

In the dye titration adding IgG, in which we observe the ligand fluorescence quenching, we used the simple single binding site model [14]:

$$Q = \frac{m[ABS]}{1/K + [ABS]} + Q_0 \tag{5}$$

where Q is the observed quenching percent, m the limit of Q at saturation, K the association constant, [ABS] the concentration of free *antibody binding sites* (ABS), and Q_0 is the constant corresponding to the observed quantity in the absence of ligand. Q was calculated by the ratio of the fluorescence intensity free of antibodies to the intensity with addition of antibodies. [ABS] was calculated by subtracting the total concentration of added antibody sites from the bound sites. The latter is equal to the bound dye concentration, and can be estimated from the fluorescence quenching by:

$$b = [TMR]_0 \frac{F_0 - F}{F_0 - F_b} \tag{6}$$

The use of Eq. (5) in the present work gives an approximate result because we are working with a polyclonal antibody.

3. Results and discussion

3.1. Spectral properties of TMR

The absorption spectrum of TMR in aqueous solution has a maximum at 552 nm and extends from 475 to 580 nm (Fig. 1a). Upon binding to anti-TMR-IgG, the absorption maximum red-shifts by 5 nm (Fig. 1a), and this reflects the fact that the IgG binding sites represent environments of much lower polarity than bulk water. Since the fluorescence maximum of TMR is located at 572 nm (Fig. 1b), the Stokes shift is 20 nm. Taking the average of the absorption and fluorescence maxima we can estimate the energy of the first singlet excited state of TMR to be 2.21 eV. The position of the fluorescence maximum and the shape of the fluorescence spectrum of TMR do not change upon binding (Fig. 1b), but the fluorescence quantum yield decreased about 60%. The position of the absorption maximum of TMR is unaffected upon binding the CD cavity (Fig. 1c).

3.2. IgG titration with TMR

In order to investigate the binding properties of TMR to the binding sites of IgG we conducted a titration adding aliquots of TMR to a fixed amount of IgG. A requisite procedure to account for the amount of bound dye was to compare the fluorescence intensity as a function of [TMR] in the absence and in the presence of IgG (Fig. 2). In the absence of IgG, the fluorescence intensity against [TMR] displays a linear behavior, which in turn, confirmed no aggregation problems in the concentration range examined. In contrast, in the presence of IgG the fluorescence intensity curve lies below the curve of the free dye, but above $[TMR] \approx 140$ nM these curves are parallel indicating the onset of saturation of the antibody. The onset of saturation, in conjunction with the fact that the antibody consumes two equivalents of the dye, served to estimate the concentration of

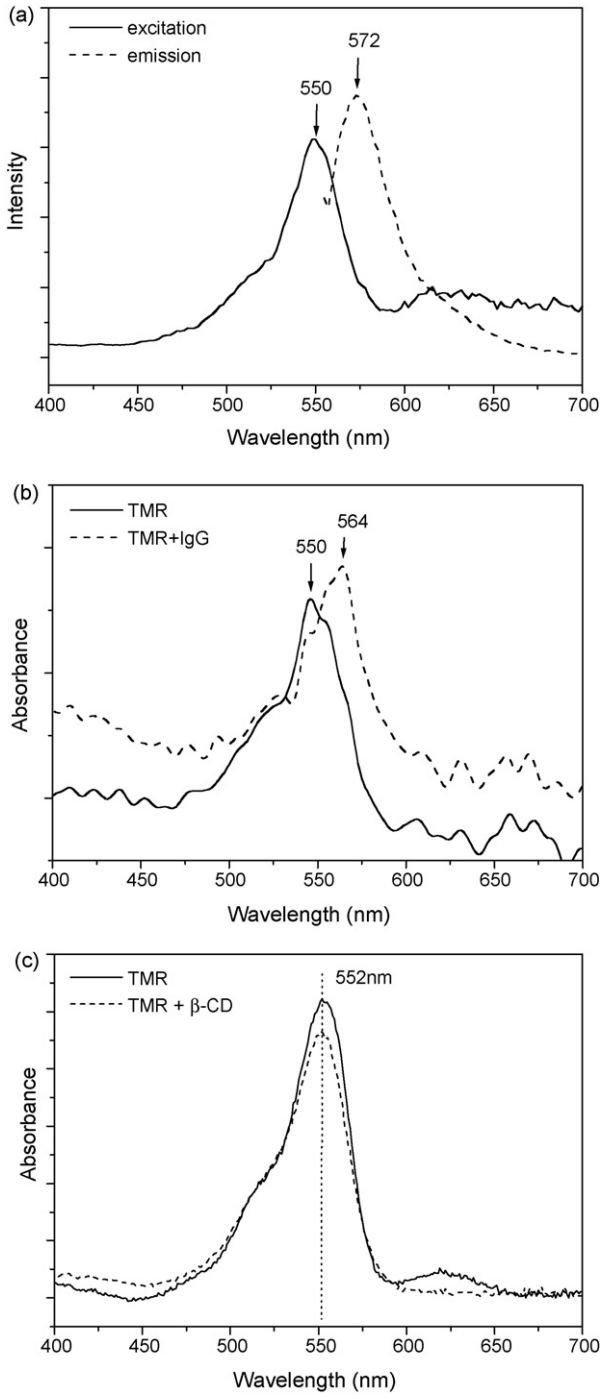


Fig. 1. (a) Absorption spectrum of free TMR and bound to IgG in phosphate buffer solution (pH 7.2), at concentration 0.3 μM. (b) Fluorescence spectra of free TMR in solution. For the emission profile, excitation was at 550 nm; for excitation profile, the emission was recorded at 572 nm. (c) Comparison of absorption spectra of free TMR and TMR associated with β-CD.

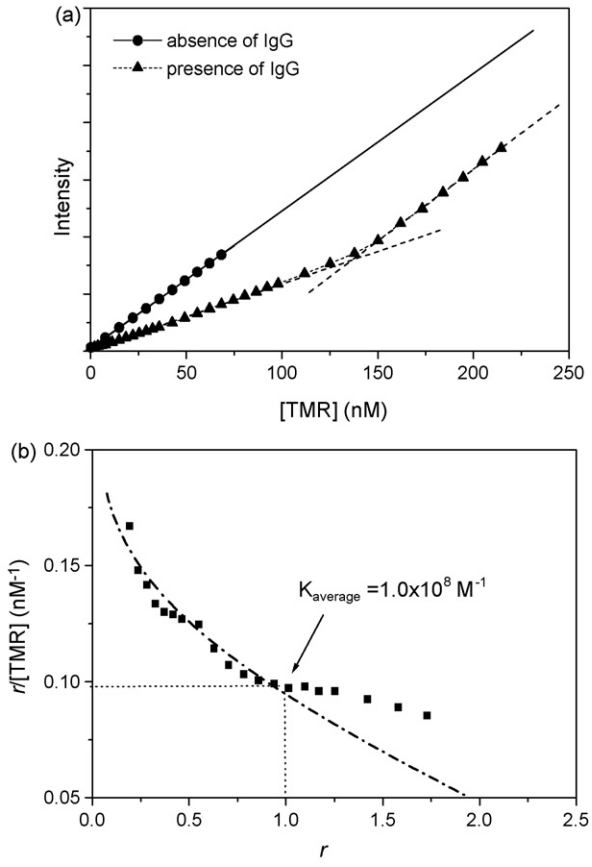
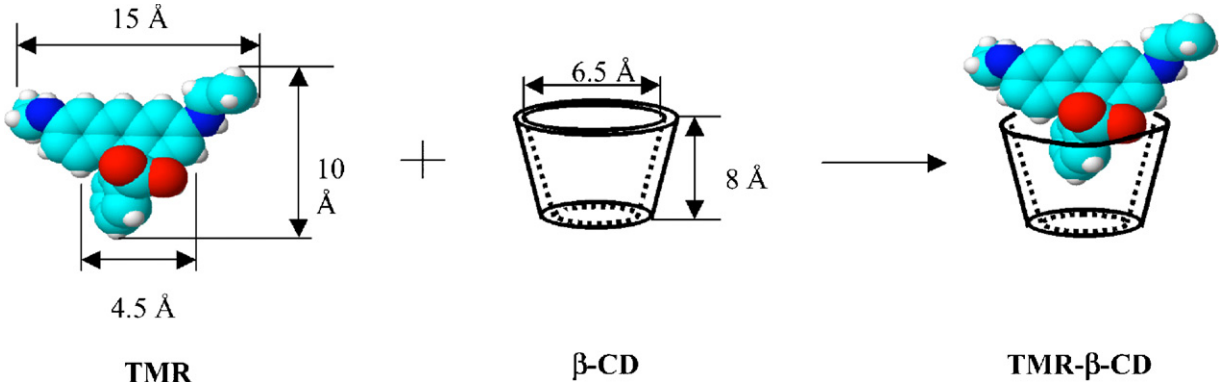


Fig. 2. (a) Titration of IgG adding TMR. The circles correspond to addition of TMR into blank buffer solution and (b) scatchard plot to estimate the binding constants according to Eqs. (1)–(4).

the antibody, [IgG] = 70 nM. In addition, we used these data to construct the Scatchard plots (see Eq. (1)) presented in Fig. 2b in an attempt to measure the binding constant. The points cannot be adjusted to a straight line because we are studying a polyclonal antibody, that consists of a mixture of IgG molecules with a significant spread in their binding affinities. However, we can still estimate an effective binding constant, $K \approx 1 \times 10^8\ \mathrm{M}^{-1}$, by extending the curve to the intercept equals two in the *r*-axis (*i.e.*, two antigen binding sites per IgG molecule). In a latter section we will present a different method to estimate this constant.

3.3. Binding of TMR to the CD cavity

In this section, we present spectroscopic and thermodynamic evidence for the formation of the TMR-CD host–guest complex. To avoid self-absorption and aggregation problems the TMR concentration was kept below 1 μM. Unlike most organic molecules, the fluorescence yield of TMR decreases upon binding the CD cavity. Employing this effect we measured the constant for the equilibrium, TMR + CD ↔ TMR-CD, at four different temperatures (listed in parenthesis), following the procedure described by El Baraka et al. [15]: $1430 \pm 70\ \mathrm{M}^{-1}$ (283 K), $1070 \pm 50\ \mathrm{M}^{-1}$ (293 K), $960 \pm 40\ \mathrm{M}^{-1}$ (298 K), $640 \pm 30\ \mathrm{M}^{-1}$ (313 K) (plots not shown). In particular, at room temperature the free energy is, $\Delta G^\circ \approx -19\ \mathrm{kJ\ mol}^{-1}$. From a van't Hoff plot we derived the values of the enthalpy and entropy associated to this process, $\Delta H^\circ = -19\ \mathrm{kJ\ mol}^{-1}$ and $\Delta S^\circ \approx 0\ \mathrm{kJ\ mol}^{-1}\ \mathrm{K}^{-1}$. The fact that the process is enthalpy driven is characteristic of host–guest complexes [8]. As opposed to the formation of a surface complex, which are entropy driven and nearly thermoneutral [16].



Scheme 1. Association of TMR to the CD cavity.

The TMR molecule is comprised of two main fragments, namely the benzyl group and the three fussed rings, joined by a covalent bond (see Scheme 1). Since the length of the three fussed rings is about 15 Å, this portion of the molecule does not fit inside the CD cavity. We can even rule out partial binding of this moiety; otherwise, the absorption band should be shifted to the red, as it was observed when TMR binds to IgG. In contrast, the benzyl moiety fits inside the CD cavity. Because this moiety does not participate in charge-transfer transitions, it is insensitive to changes in polarity. This is consistent with our experimental observation that the position of the maximum of the TMR absorption band did not shift upon binding (Fig. 1c).

3.4. TMR titration with IgG in the presence of CD

As mentioned above, the fluorescence yield of TMR decreases, although to different extends, upon binding to either the CD pocket or to the IgG antigen binding sites. Here, we describe an experiment that clarifies this point. Fig. 3a shows that the TMR fluorescence decreases steadily upon adding CD until $[CD] \approx 10^{-2}$ M, which is slightly below the maximum amount of CD that can be dissolved in water at room temperature. The fluorescence decreases even more upon adding IgG up to a concentration of $[IgG] \approx 70$ nM. On the other hand, adding IgG first, up to the maximum concentration reached in the experiment shown in Fig. 3a, the fluorescence intensity remains the same even at the highest concentration of CD. Nonetheless, the overall fluorescence decrease in both experiments was the same.

Further insight into these equilibria was obtained by titrating IgG with a solution of TMR mixed with different amounts of CD. Close examination of Fig. 4a reveals that the quenching of the TMR fluorescence becomes less efficient upon increasing the CD concentration. Fig. 4b displays the dependence of the quenching percent Q for different CD/IgG concentration ratios. We employed Eqs. (5) and (6) to fit these data, from which we derived the apparent association constants. The binding constant estimated in the absence of CD (sample #1), $K \approx 2.5 \times 10^8\ M^{-1}$, is in good agreement with the average $K \approx 1.0 \times 10^8\ M^{-1}$ previously estimated from the Scatchard analysis. The magnitude of the association constant reduces to one fifth of its original value at the highest CD concentration examined. This result in conjunction with the spectroscopic evidence that suggests that IgG and CD interact with different TMR moieties, suggest that TMR is binding simultaneously to IgG and CD.

3.5. Fluorescence anisotropy measurements of TMR complexes

We measured fluorescence anisotropy to obtain more information about the binding properties of TMR to IgG [17–19]. The steady-state anisotropy of TMR in water ($\eta = 0.94$) was $r = 0.05$

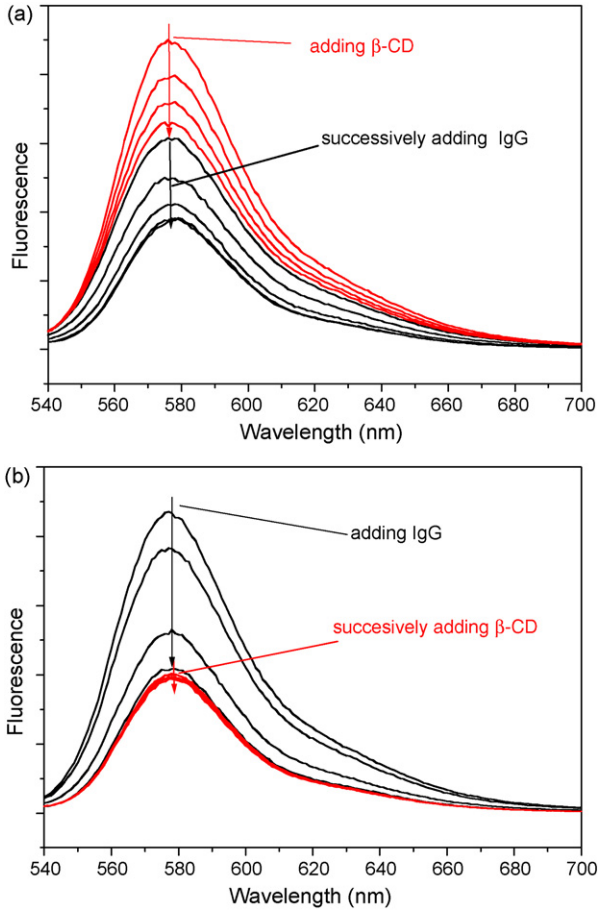


Fig. 3. Fluorescence spectra obtained under the following conditions: (a) adding first CD followed by the addition of IgG and (b) IgG is first added, then CD is added.

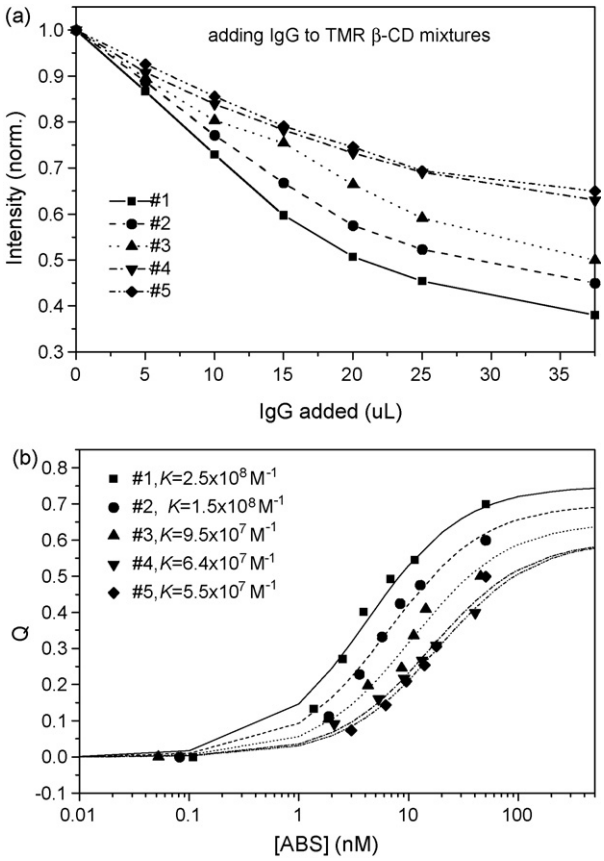


Fig. 4. (a) Percent quenching upon adding TMR mixed in different proportions with CD. (b) A heuristic fitting of quenching TMR + β-CD with anti-TMR antibody solution (ca. 1 mM) according to Eqs. (5) and (6). The concentrations arrangements for five mixture samples #1–5 as follows: initial $[TMR]_t$ is 30 nM for all samples, with $[\beta\text{-CD}]_t$ of $4 \times n$ mM ($n = 0$–4 for #1–5, respectively).

(*i.e.*, nearly zero), as expected for a low molecular weight fluorescent molecule in a fluid of low viscosity. In contrast, in a solvent of high viscosity such as glycerol ($\eta = 1500$ cP) the measured anisotropy was $r_0 = 0.37$. For a non-rotating molecule with the absorption transition vector (μ_a) oriented at an angle γ with respect to the emission transition vector (μ_a), the anisotropy is given by, $r_0 = (3\cos^2\gamma - 1)/5$. The limiting anisotropy measured in glycerol confirms that in the TMR molecule the vectors μ_a and μ_e are orientated nearly perpendicular [20]. In other words, the dynamic range in experiments in which the anisotropy of TMR is measured is, $0 \leq r \leq 0.4$.

In the presence of $[CD] = 10^{-2}$ M, the anisotropy of TMR was also nearly zero, in spite the fact that the molecular mass of the complex was about three times heavier than that of the free TMR molecule. Considering that the binding constant of TMR to the cavity of CD at room temperature was $1070 \pm 50\ M^{-1}$ and that the concentration of CD in our experiment was more than two orders of magnitude larger than [TMR], we are confident that the TMR + CD ↔ TMR-CD equilibrium was shifted to the right (*i.e.*, the TMR-CD inclusion complex was the most abundant form of TMR). A null anisotropy may indicate that TMR is not bound tightly to the CD cavity, namely TMR it is not following the rotational motion of the CD molecule. Another plausible explanation is that the dissociation time of the TMR-CD inclusion complex is much shorter than its correlation rotational time.

We took advantage that TMR is also fluorescent when it is bound to IgG to measure the anisotropy of the TMR-IgG complex, $r = 0.28$. Since this value of anisotropy is smaller than $r = 0.4$, it reflects the mobility of TMR within the antigen binding site. As the work of Yguerebide et al. demonstrated, monitoring time resolved-fluorescence anisotropy, it is possible to resolve different local rotational motions [7]. Because in our work we recorded steady-state anisotropy, we were unable to disentangle the different rotational motions.

4. Concluding remarks

In this work, we estimated the magnitude of the binding constants of TMR to an antibody specific for this dye, $K \approx 10^8\ M^{-1}$, using two different methods. We observed a pronounced decrease in the magnitude of the binding constants upon adding CD. Experimental evidence suggests that TMR can interact simultaneously with the IgG antigen binding sites and with the CD cavity. The smallness of the anisotropy displayed by the TMR-IgG complex indicated that TMR was not bound rigidly to the IgG binding site; instead, our data suggested that the dye was undergoing local depolarizing motions. These observations establish a clear distinction between an antigen that binds to IgG with either high or else small affinity and those that bind rigidly.

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