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Fluorescence Spectroscopy of Monoclonal Antibodies Produced Against the Fluorescyl Hapten Conjugated Through the Xanthene Ring

P. R. Droupadi,[1] T. Nanavaty,[1] C. Smith,[1] D. D. Johnson,[2] M. Adamczyk,[2] and D. S. Linthicum[1,3]

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Two mouse anti-fluorescyl monoclonal antibodies (mAb), clones FL43.1 and FL55.3, were produced to the fluorescein hapten, which was conjugated to the carrier protein through the 4' position of the xanthene ring. Association constants (K_A) and thermodynamic parameters for both mAb were ascertained by monitoring the steady–state intrinsic and fluorescein fluorescence. Both techniques were in good agreement and gave K_A values in the 10^9 M^{-1} range. Ligand–induced intrinsic fluorescence quenching showed a hypsochromic shift for mAb FL43.1, but not for FL55.3, suggesting that the ligand interacts with different tryptophan residues in each mAb. Because these mAb are directed toward the phenylcarboxylate portion of fluorescein, the different ionic and structural forms should be useful as indicators of antibody binding site pH and buffering capacity near the binding site.

KEY WORDS: Monoclonal antibody; fluorescein; complex; fluorescence quenching; intrinsic fluorescence.

INTRODUCTION

The binding interaction between an antibody and antigen can be described as a "molecular complexation" process, in which a combination of thermodynamic, geometric and chemical parameters define the basis of antigen recognition and binding.[1] Empirical studies of antibody–antigen complexes involving X-ray diffraction of crystals have revealed that the majority of the contact or interactive residues in the antibody are localized in the hypervariable loop regions of the Fab (fragment antigen binding) portion of the antibody.[2] In the Fab molecule there are six hypervariable loops, or so-called complementarity-determining regions (CDR),[3] three derived from each heavy (H) and light (L) polypeptide chain.[4]

Although the amino acid sequences and lengths of the six CDR may vary considerably from one antibody to another, and such differences are generated by gene selection and gene recombination events,[5] there is a propensity for the occurrence of specific residues and loop shapes in certain positions of the CDR.[6,7] Chothia and Lesk[8,9] were first to note the canonical structural features of the CDR and attributed these to the presence of particular amino acids in key positions and their ability to assume unusual peptide dihedral angles. They concluded that known antibody structures showed a limited number of backbone conformations based on these canonical features, and thus the overall architecture of these different antibodies, despite their differing antigen specificities, was conserved.

It has also been noted that antibody binding sites

[1] Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843-4467.
[2] Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, Illinois 60064.
[3] To whom correspondence should be addressed.

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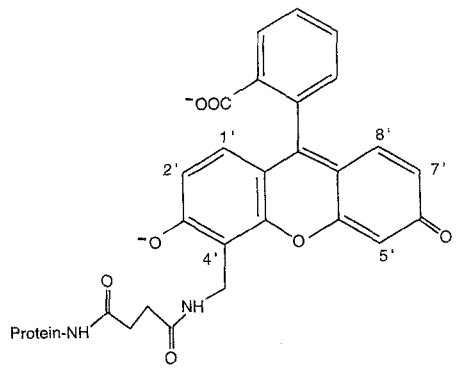

Fig. 1. Structure of fluorescein hapten conjugated through the 4' position to the carrier protein (bovine serum albumin). The dianion form (pK_{COOH} = 4.4, pK_{OH} = 6.7) of fluorescein is depicted.

have an unusual propensity for certain types of amino acids in the CDR, such as Arg, Trp, and Tyr.[6] Large aliphatic residues, in sharp contrast, are rarely found in the CDR,[10] and this may be due to their hydrophobic nature, as well as the energetic penalty, due to torsional constraints of the side chains, upon antigen binding. In contrast, aromatic residues in the binding site appear to contribute significantly to the specificity and stability of antibody–antigen complexes.[11–13] The ability of aromatics to participate in a combination of interactions, such as H bonding, hydrophobic contacts, and electrostatics (e.g., π–π stacking, charge transfer) make them favorable residues for antigen binding.[14,15]

The intrinsic fluorescence of Tyr and Trp residues in the antibody CDR provides a useful feature for analyzing the antibody–antigen complexation process.[16] In many instances, antigen binding may quench the fluorescence of the binding site aromatics by direct contact, alteration of the dielectric constant of the surrounding environment, or energy transfer.[17] If the antigen itself possesses fluorogenic properties, these may also be altered during the binding process. To take advantage of this in a direct fashion, a number of investigators have used fluorescent compounds as antigens. Fluorescyl, dinitrophenyl, and dansyl derivatives are among the most popular fluorogenic haptens.[18–21] As in most cases, such low-molecular-weight haptens must be first chemically conjugated to a larger protein carrier to render them immunogenic. As a result of this covalent conjugation process, the resulting antibodies have the majority of their specificity directed toward the atoms distant to the covalent linkage site. For the hapten fluorescein all antibodies reported have been made to the hapten conjugated through the 5- or 6-position of the phenylcarboxylate, thereby resulting in antibodies that predominately recognize the xanthene ring of the ligand.[21] The most popular derivatives used are the 5- and 6-fluorescein isothiocyanate, 5- and 6-carboxyfluorescein, and 5- and 6-aminofluorescein. The best-characterized mAb of this type studied to date, clone 4-4-20, binds the entire fluorescein molecule in the binding pocket.[22]

We have produced a panel of monoclonal antibodies against the fluorescein ligand conjugated through the 4'-position of the xanthene ring, so that the immunodominant portion of the ligand is the phenylcarboxylate group. We report here the spectroscopic properties of two anti-4'-fluorescyl mAb and their complexes with the hapten fluorescein. We examined the thermodynamic parameters of both intrinsic tryptophan and fluorescein fluorescence quenching.

MATERIALS AND METHODS

Monoclonal antibodies FL43.1 and FL55.3 were produced to the hapten fluorescein conjugated through the 4'-position of the xanthene ring (4'-(aminomethyl)fluorescein[23–25]) to bovine serum albumin carrier protein (Fig. 1), using standard procedures developed in our laboratory.[26] The mAb were purified by protein-G affinity chromatography and the purity was verified by gel electrophoresis.[26]

All mAb and ligand solutions were made in 0.01 *M* phosphate-buffered saline (0.15 *M* NaCl) (PBS), pH 7.4, and filtered with a 4-μm-filter. The quartz cuvettes were siliconized (Sigmacoat, Sigma Chemical Co., St. Louis, MO) prior to use, to avoid adsorption of protein. All stock solutions were stored at 4°C in the dark.

Absorption spectral measurements were carried out on a Beckman DU650 spectrophotometer using a 0.1 μ*M* solution of fluorescein titrated with a 14 μ*M* solution of mAb. This concentration was selected for the purposes of detecting the charge-transfer band and isosbestic points. The emission spectroscopic studies were carried out using an SLM8100 spectrofluorimeter equipped with a temperature-controlled cuvette holder. The solutions in the cuvettes were stirred constantly with a magnetic stirring bar. Excitation and emission wavelengths for fluorescein were 470 and 512 nm, respectively. The emission scan for the mAb was carried out in the 315- to 365-nm range. The excitation and emission wavelengths chosen for the mAb were 280 and 335 nm, respectively. The excitation at 280 nm was chosen to optimize the emission signal. Excitation and emission band pass were kept at 4 and 8 nm. The relative fluorescence intensities were measured as a ratio of the fluorescent beam to a reference beam to correct for any

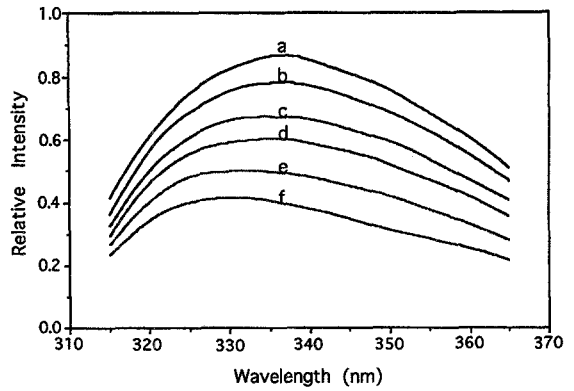

Fig. 2. Emission spectrum of 5.0 n*M* mAb FL43.1 alone (a) and in the presence of fluorescein at the concentrations 5.53 n*M* (b), 16.6 n*M* (c), 38.6 n*M* (d), 71.6 n*M* (e), and 115 n*M* (f), in PBS at 25°C.

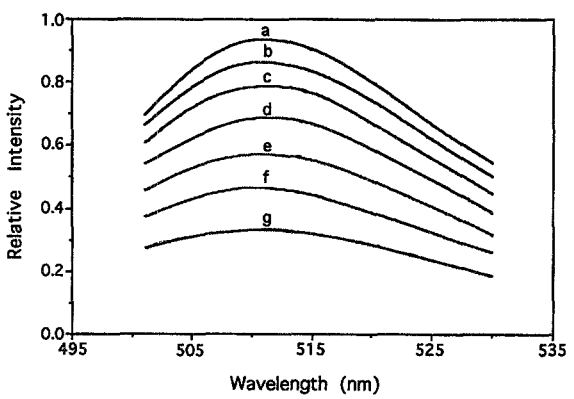

Fig. 3. Emission spectrum of 2.0 n*M* of fluorescein alone (a) and in the presence of mAb FL43.1 at the concentrations 0.33 n*M* (b), 0.72 n*M* (c), 1.20 n*M* (d), 1.77 n*M* (e), 2.44 n*M* (f), and 3.21 n*M* (g) in PBS at 25°C.

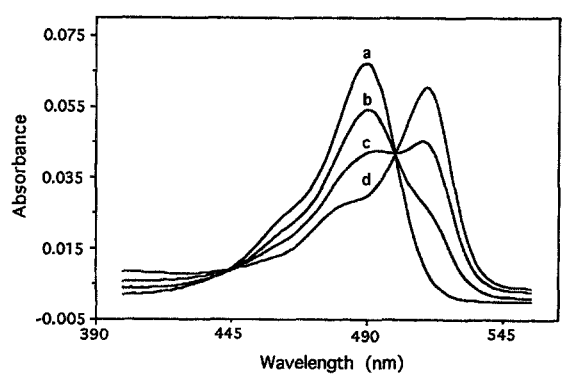

Fig. 4. Absorption spectra of 0.9 μ*M* of fluorescein alone (a) and in the presence of mAb FL43.1 at concentrations of 0.91 μ*M* (b), 2.10 μ*M* (c), and 4.08 μ*M* (d), in PBS at 25°C.

fluctuations in lamp intensity. The photomultiplier voltage was maintained below 1000 V to increase the signal-to-noise ratio. The emission intensities were collected during 10-sec intervals at 335 nm.

A typical titration was carried out with the addition of 5–20 μl of a μ*M* solution of fluorescein or mAb using a Hamilton microsyringe to 3.0 ml of PBS to yield a 10 n*M* solution. All of the solutions were equilibrated for 90 min at set temperatures prior to spectral measurements. Photobleaching effects were not observed for the mAb at the concentrations used in this study.

The data were analyzed according to the method of Stinson and Holbrook[27] using a curve-fitting procedure: $1/(1 - \theta)K_A = [X_T]/\theta - p[A_T]$, where θ, the fractional occupancy of binding sites, is given by $F_0 - F/\Delta F_{max}$, with F_0 the emission intensity of mAb in the absence of ligand and F is that in the presence of ligand. K_A is the intrinsic association constant, p is the number of binding sites, X_T is the concentration of ligand, and A_T is the concentration of the mAb.

To measure the thermodynamic parameters, ln K_A was plotted against $1/T$ at different temperatures (slope $= -\Delta H/R$). The change in free energy of the system was obtained from the equation $\Delta G = -RT \ln K_A$. The change in entropy of the system was obtained from the equation $\Delta G = \Delta H - T\Delta S$.

RESULTS

Intrinsic tryptophan quenching of mAb FL43.1 and FL55.3 could be observed upon binding of the ligand fluorescein. As shown in Fig. 2, a hypsochromic shift in the quenched emission spectra was observed for mAb FL43.1. The emission maximum of the native antibody was at 337 nm and blue shifted at ligand saturation. Quenching of the fluorescein ligand fluorescence did not show a shift in the quenched spectra (Fig. 3). For mAb FL55.3 the fluorescence quenching for both tryptophan and fluorescein emission did not show a spectral shift.

The absorption spectra for fluorescein were found to be significantly red shifted (27 nm) upon complexation with both mAb (Fig. 4). The presence of isosbestic points indicated a 1:1 stoichiometric complexation for both mAb FL43.1 and FL55.3. We were unable to examine these complexes for the presence of a charge-transfer band of the mAb, due to the overlaps in spectra for the mAb and fluorescein in the 320- to 350-nm range.

Stinson–Holbrook analysis was used to determine the association constants. Figure 5 shows the plot of $1/(1 - \theta)$ vs. ligand concentration/θ for mAb FL43.1 at 30°C.

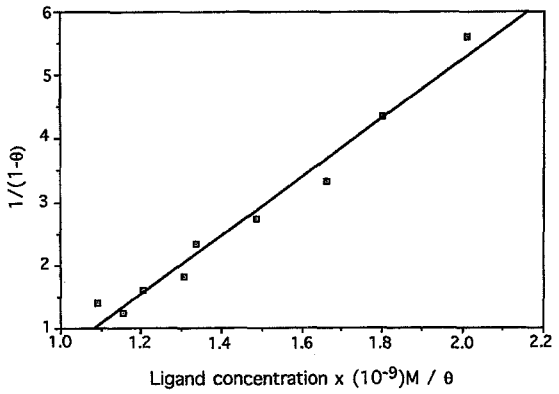

Fig. 5. Stinson–Holbrook plot of $1/(1 - \theta)$ versus ligand concentration/θ for the interaction of mAb FL43.1 with fluorescein in PBS at 32°C.

Table I. Intrinsic Association Constants and Thermodynamic Parameters of mAb FL43.1 and FL55.3 at 25°C in PBS

Fluorescence monitored	Quencher	K_A ($\times 10^9$ M^{-1})	ΔG (kcal M^{-1})	ΔH (kcal M^{-1})	ΔS (cal M^{-1} K^{-1})
FL43.1	Fluorescein	2.24 ± 0.15	−12.68	−4.04	28.98
Fluorescein	FL43.1	1.58 ± 0.11	−12.54	−7.62	16.52
FL55.3	Fluorescein	1.41 ± 0.10	−12.47	−8.67	12.80
Fluorescein	FL55.3	1.84 ± 0.12	−12.63	−10.03	8.73

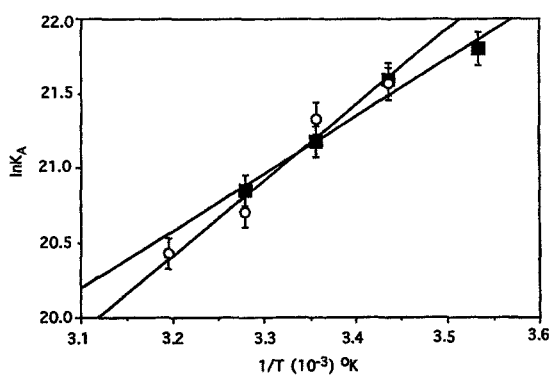

Fig. 6. Van't Hoff plot of ln K_A versus $1/T$ (K) for mAb FL43.1 (■) and FL55.3 (○).

The K_A values obtained by this method were the mean values from three repetitive determinations (Table I). The K_A values determined using the tryptophan quenching were in good agreement with those derived from fluorescein quenching measurements. To calculate, the free energy change upon complexation, a van't Hoff plot of ln K_A vs. $1/T$ was generated for both mAb (Fig. 6). The slope of the plot corresponds to the enthalpic change involved in the reaction. Both mAb showed positive slopes, indicating that the interactions were exothermic. The entropy changes were determined to be small relative to the enthalpic values.

DISCUSSION

Aromatic amino acid residues in proteins, especially tyrosine and tryptophan, are known to be abundant in antibody variable regions and, in particular, within the CDR.[6] Their participation in antigen recognition and binding has been noted for several antigens in empirical Fab–antigen complexes solved by X-ray diffraction[2] and NMR techniques.[11,28] Aromatic residues known to be in contact with antigen appear to provide a variety of robust interactive motifs. These residues can participate in van der Waals contacts, hydrogen bonding, π–π stacking, and aromatic–cation interactions.[29]

Spectroscopy of most antibodies reveals the maximal emission wavelengths between 330 and 340 nm, and the majority of this fluorescence is due to tryptophan emission; contributions by tyrosine and phenylalanine tend to be smaller due to their lower quantum yields.[30] Although there are numerous conserved tryptophan residues in the framework of the Ig structure, many of these are in buried positions (e.g., L:35W, H:36W, H:103W) and their fluorogenic properties are reduced and blue shifted due to contacts with other residue side chains and the low dielectric constant of the immediate environment.[31] In contrast, tryptophan residues found in the CDR (e.g., L:96W) are often exposed to solvent and can occupy different geometric orientations, depending upon the state of the protein and the local environment. Upon antigen complexation, however, the fluorogenic properties of such contact residues change, and the measurement of these changes (usually quenching) can be a useful monitor of the antibody–antigen complexation process.[31] In addition, the relative exposure to the solvent and position of specific tryptophan residues can be assessed by shifts in the emission maxima. If a solvent-exposed tryptophan residue is quenched in a selective manner by ligand interactions, then the remaining fluorescence will be due to buried tryptophan residues, which tend to be blue shifted relative to the solvent-exposed population.

In this study we measured the steady-state quenching of both intrinsic fluorescence (due to mAb Trp) and fluorescence quenching of the ligand fluorescein in two different mAb. For both mAb studied here the association constants ($K_A = 2 \times 10^9$ M^{-1} range) were deter-

mined using Stinson–Holbrook analysis for both types of quenching measurements and found to be in good agreement. We examined the binding and calculated the association constants over a temperature range of 10–32°C, but did not observe curvilinear van't Hoff plots;[32] it can be concluded from these data that the changes in enthalpy were temperature independent. These observations are in distinct contrast to those made for anti-fluorescein mAb 4-4-20.[32] The emission spectrum of mAb FL43.1 showed a hypsochromic shift upon ligand binding, suggesting that a solvent-exposed Trp residue in the binding site is quenched by the ligand. In addition, the absorption spectrum for fluorescein was found to have a red shift which is indicative of a charge-transfer interaction.[33] However, due to the absorption of fluorescein in the 320- to 350-nm range, we were unable to identify directly the presence of a charge-transfer band of the mAb.

The strong fluorescence of fluorescein is primarily due to the xanthene ring. Its emission is maximal in alkaline solution and greatly reduced at acidic pH. The pH dependence of fluorescein emission is complex due to the formation of different species of the dye.[34] The mAb in this study are directed toward the phenylcarboxylate of the ligand fluorescein and this was accomplished by preparation of the immunogen linkage through the 4' position of the hapten. Others have produced polyclonal and monoclonal anti-fluorescyl antibodies by directing the response to the xanthanoyl ring by conjugation via the 5- or 6-position of the phenylcarboxylate group using fluorescein isothiocynate.[22] The mAb examined in this study should be sensitive to the different structural forms of fluorescein, such as the neutral lactonic and quinonoid forms, as well as the monoanionic form.[35] These different fluorescein forms should prove useful as indicators of the antibody-binding-site pH, and buffering capacity in and around the antibody binding site.

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