Fluorescence measurements of immune complexes of Mab 4-4-20 with isomeric haptens

Cathy A. Swindlehurst and Edward W. Voss, Jr.
Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801 USA

ABSTRACT Relative differences in the active site environment of a monoclonal antibody when covalently bound to two isomeric haptens were studied using fluorescence quenching and lifetime measurements. Murine monoclonal antibody 4-4-20, a well-characterized high affinity antifluorescein antibody, served as the model IgG protein. Isomeric haptenic probes comparatively studied were fluorescein-5-isothiocyanate (FITC I, the immunogen) and fluorescein-6-isothiocyanate (FITC II). In kinetic binding studies, the association rate for the interaction of 4-4-20 with FITC I was >2,000 times faster than the reaction with FITC II. Fluorescence lifetimes for FITC I covalently bound to 4-4-20 were 3.89 ns and 0.37 ns, indicative of hapten bound outside and inside the active site, respectively. Fluorescence lifetime for FITC II within the active site was indistinguishable from bound FITC I, indicating that interactions with active site residues which resulted in a decreased lifetime were similar for both isomers. A decreased lifetime for active site bound FITC I was consistent with the 90–95% quenching of fluorescein fluorescence. Dynamic fluorescence quenching experiments with iodide and FITC I in the active site showed no solvent accessibility, whereas bound FITC II showed significant accessibility. These results suggest that the difference in bond angle which accompanies binding of isomer II relative to isomer I within the active site probably leads to steric constraints resulting in a more open configuration of the 4-4-20 active site.

INTRODUCTION

In pursuit of a better understanding of antigen binding properties exhibited by immunoglobulin products of humoral immune responses, and in particular the determining factors in high affinity, attention has focused on understanding the nature and environment of the antibody active site. Because naturally occurring immunogens are often complex macromolecules, whose structure and conformation are usually not well defined, it has been difficult to ascertain critical factors involved in such antigen-antibody interactions. For this reason hapten systems, in which the antigen is usually a relatively small compound (mol wt $\leq$ 1,000) of defined structure, serve as valuable experimental tools.

Haptens can be judiciously selected to provide desired properties such as immunogenicity, size, aromatic and/or ionic character, and observable spectral or even chiral properties. Because fluorescence measurements are sensitive and reflect the fluorophore's microenvironment, fluorescent haptens have become important probes. In addition to intensity measurements, one measurable fluorescence parameter which provides useful information is the fluorescence (or excited-state) lifetime. Relevant biological events which occur on the timescale of fluorescence lifetime (picoseconds to hundreds of nanoseconds) include molecular rotation and interactions with solvent molecules and neighboring residues (1).

Fluorescence measurements provide an intrinsic sensitivity to monitor the environment surrounding the fluorophore and has led to extensive use of fluorescence to establish existence of multiple conformational states in proteins (1). When applied to small proteins such measurements have generally utilized the intrinsic fluorescence of a single tyrosine or tryptophan residue per macromolecule. Application of fluorescence lifetime measurements to analyses of single tryptophan containing proteins has been described in detail in a series of papers by Alcala et al. (2–4) and Beechem and Brand (1). Antibody molecules are relatively large macromolecules (150 kD) which contain multiple tyrosine and tryptophan residues negating the application of this approach. To avoid this restriction, it is often necessary to utilize extrinsic fluorescent probes. Consistent with this rationale the fluorescein:antifluorescein system, in which fluorescein is both the antigen and an extrinsic fluorescent probe, has become an important model system to derive information concerning the microenvironment of the antibody active site (5).

This work employed fluorescence measurements to probe the active site of monoclonal antifluorescein antibodies. When conjugated to an appropriate carrier, fluorescein is a potent epitope which elicits a diverse immune response in several species, including signifi-

Address correspondence to Dr. Edward W. Voss, Jr.

cantly high affinity antibodies (6). Spectral properties of fluorescein, which make it a useful probe, include absorbance in the visible region of the spectrum (493 nm), a reasonable extinction coefficient (72,000 M^{-1} cm^{-1}), excitation and emission maxima (493 and 525 nm, respectively) which do not overlap with intrinsic protein fluorescence (350 nm), and a large quantum yield (>90%). Spectral shifts in absorbancy and quenching of fluorescence upon antibody binding of fluorescein provide information regarding mechanisms involved in ligand binding (5).

A homogeneous antifluorescein antibody used in this work was the high affinity ($1–2 \times 10^{10}$ M^{-1}) murine monoclonal antibody (Mab) 4-4-20. Mab 4-4-20 has undergone extensive characterization in terms of spectroscopic and binding properties (7) as well as primary structure (8). In addition the three-dimensional crystal structure has been solved for the 4-4-20-fluorescein complex (9, 10). Recently, Mab 4-4-20 served as a model protein in the direct observation of immunoglobulin adsorption dynamics using the atomic force microscope (11).

Studies reported include absorbance measurements and fluorescence lifetime and quenching measurements of Mab 4-4-20, *N*-acetyl-L-lysine and normal mouse IgG covalently labeled with fluorescein-5-isothiocyanate (FITC I) or fluorescein-6-isothiocyanate (FITC II). Although Mab 4-4-20 is referred to generically as an antifluorescein antibody, it is more precisely antiFITC I because the original immunogen was synthesized using fluorescein-5-isothiocyanate. Thus, availability of the same spectral probe (fluorescein) in isomeric forms provided a unique opportunity to couple with sensitive fluorescent measurements in a comparative study of the homologous isomer (I) and a cross-reactive isomer (II). Affinity labeling with FITC isomers I and II provided two active site probes with identical spectral properties but with the potential to bind differently within the active site. It was the goal of this work, based on two isomers of the same ligand, to provide information that contributes additional insight into the chemical nature, conformation, and dynamics of the antibody active site.

MATERIALS AND METHODS

Cell line

Hybridoma cell line 4-4-20 was obtained by chemical fusion of the nonsecreting murine myeloma cell line Sp2/0-Ag 14 with splenocytes from an inbred colony of BALB/cV mice hyperimmunized with fluorescein isothiocyanate isomer I (FITC I) conjugated to keyhole limpet hemocyanin (KLH) and emulsified in complete Freund's adjuvant as described by Kranz et al. (12) using the method of Galfre et al. (13).

Production of monoclonal antibody protein

Mab 4-4-20 was produced in serum free media in an airlift bioreactor (14). After removal of cells and concentration of extracellular media, the antibody was purified using preparative protein-A affinity chromatography (14). Immunoglobulin purification was accomplished by dextran sulfate precipitation of serum lipoproteins and ammonium sulfate precipitation of the gamma globulin fraction followed by DEAE-cellulose ion-exchange chromatography at pH 7.4. Affinity purification with a F1-adsorbent was avoided because acid or high salt elutions of Mab 4-4-20 resulted in release of some ligand (from the adsorbent) which was difficult to quantitatively remove due to tight binding. Mab 4-4-20 has an intrinsic affinity (K_a) of $1–2 \times 10^{10}$ M^{-1} and consists of kappa (κ) light and gamma (γ) 2a heavy chains. A red shift in the wavelength of maximum absorbance (λ_{MAX}) of fluorescein from 493 to 508 nm and a maximum quenching of the fluorescence of fluorescein (Q_{MAX}) of 96.4% upon binding of fluorescein by 4-4-20 has been reported (6, 7).

Normal mouse IgG

Young nonimmunized mice were bled as a source of normal IgG to be used as a control reagent. Immunoglobulin purification was accomplished by dextran sulfate precipitation, ammonium sulfate precipitation, and diethylaminoethyl (DEAE) cellulose chromatography as previously described.

Ig sample preparation

For labeling reactions, IgG was diluted to 1 mg/ml with buffer. FITC labeling reactions were performed by adding appropriate amounts of 10^{-3} M FITC (Molecular Probes, Inc., Eugene, OR) in absolute ethanol to 1–2 ml of the diluted immunoglobulin (corresponding to 13.3 μl of 10^{-3} M FITC in 2 ml of IgG at a concentration of 1 mg/ml). Samples were incubated at room temperature for 2–3 h and dialyzed extensively (minimum of six buffer changes over three days) against 50 mM Tris(tris[hydroxymethyl]-aminomethane), 150 mM sodium chloride (TBS) buffered at pH 8.3.

FITC-*N*-acetyl-L-lysine was prepared by incubation of *N*-acetyl-L-lysine with excess FITC (I or II) in carbonate buffer for 2–3 h. FITC-*N*-acetyl-L-lysine was purified by preparative TLC on silica gel using methylethylketone saturated with acetate buffer (pH 5.0) as the solvent. A visible band containing fluorescein labeled *N*-acetyl-L-lysine was harvested from the plate and solubilized in buffer.

Instrumentation

A model 8450A (Hewlett-Packard Co., Palo Alto, CA) Diode Array spectrophotometer was used in absorbance measurements. Fluorescence intensity measurements were performed on a photon counting spectrofluorometer (Gregg-P.C., ISS Instruments, Champaign, IL) using a xenon arc lamp with excitation at 493 nm and emission at 530 nm. A 520 nm cut-on filter was used to select emission wavelengths >520 nm. Fluorescence lifetimes were measured on the multifrequency phase fluorometer described by Gratton and Limkeman (15) using the 442 line of a HeCd laser and an acousto-optic modulator as described by Piston et al. (16). A 440 nm bandpass filter was used for excitation and 520 nm cut-on filter was used for emission. Measurements were taken using a scatter solution of glycogen as a reference. Phase and modulation data were collected using 8–12 frequencies ranging from 1 to 150 MHz.

Association rate assays

Second-order association rates of FITC I and II binding to Mab 4-4-20 were determined by monitoring the decrease in fluorescence intensity as a function of time after mixing (17). Equal volumes of 4-4-20 and FITC were mixed using a manual stopped flow apparatus (Hi-Tech Scientific model SFA-II Rapid Kinetics Assembly, Hi-Tech Scientific, Wilts, England) thermostated at 2°C. Based on the concentration of FITC used (2.0×10^{-9} M), slightly more than a 10-fold molar excess of 4-4-20 (2.7×10^{-8} M IgG) ensured first order kinetics. Fluorescence intensity measurements were taken as a function of time using kinetics software (ISS Inc., Champaign, IL). Data were saved as ASCII files and converted to comma separated variable (CSV) files for use with OnRate analysis software (University of Illinois at Urbana-Champaign, Urbana, IL). The technical rate constant (K_{tech}) was determined from the slope of the plot of the fraction free ligand (L/L_o) as a function of time (t). The fraction free ligand was calculated using the equation

$$L/L_o = \ln \frac{F(t) - F(eq)}{F(0) - F(eq)}, \quad (1)$$

where $F(t)$ is the fluorescence intensity at time t, $F(eq)$ is the equilibrium fluorescence intensity, and $F(0)$ is the initial fluorescence intensity. The bimolecular quenching constant (K_1) was calculated using the equation

$$K_1 = \frac{f_b(eq) \times K_{tech}}{[sites]}, \quad (2)$$

where $f_b(eq)$ is the fraction of bound ligand at equilibrium, [*sites*] is the concentration of active sites, and K_{tech} is the slope of the plot of L/L_o vs. t. Experiments were repeated at least in triplicate.

Fluorescence lifetime measurements

Lifetime measurements were taken (room temperature) until the error in the phase angle was less than or equal to 0.20 and the modulation ratio less than or equal to 0.01.

Lifetime analysis

Analysis of multifrequency phase and modulation data was performed on a personal computer using the Global Unlimited analysis software described by Beechem and Gratton (18). Data were analyzed using different fitting routines and in all cases the best fit was obtained for a two discrete component exponential decay.

Iodide quenching

Fluorescence quenching studies with iodide were performed as described by Watt and Voss (19).

RESULTS AND DISCUSSION

Affinity labeling control reactions

Affinity labeling of Mab 4-4-20 with FITC (I or II) involved covalent conjugation of the isothiocyanate group in the antibody active site. Crystal data defining the 4-4-20 three-dimensional structure have indicated that the most likely site of FITC I attachment is lysine residue 52b of the heavy chain (8, 10). Characterization studies of free FITC and FITC covalently attached *N*-acetyl-L-lysine and normal mouse IgG were used to differentiate active site effects from those caused by covalent binding of FITC to L-lysine, or nonactive site lysyl residues.

Absorbance measurements

A parameter denoting fluorescein binding by Mab 4-4-20 is a characteristic bathochromic shift in the maximum absorbance (λ_{MAX}) by bound ligand. To determine relative spectral shifts, absorbance measurements were performed with FITC I and II free in solution and covalently bound to N-acetyl-L-lysine, normal mouse IgC, and Mab 4-4-20 (Table 1). Because wavelength resolution limits of the absorbance spectrophotometer were 1 nm in the ultraviolet range and 2 nm in the visible range, absorbance values obtained are ±1 nm for antibody and ±2 nm for FITC.

No differences were observed in λ_{MAX} either between the two FITC isomers, FITC free in solution or conjugated to N-acetyl-L-lysine. Thus, there were no inherent spectral differences between the FITC isomers and formation of the thiocarbamyl linkage (isothiocyanate group and the ε-amino group of a lysine residue) did not result in a spectral shift. FITC I bound to normal mouse IgG exhibited a small spectral shift (4–6 nm) and labeled with an efficiency of ~40%. Mab 4-4-20 was labeled in

TABLE 1 Comparative absorbance values for FITC isomers I and II

Sample	λ_{max}	$A\lambda_{max}$	A_{278}*	r‡	*Eff.*§	λ_{max}‖
FITC I	488	0.12	—	—	—	—
FITC II	488	0.11	—	—	—	—
N-Acetyl-L-Lysine						
FITC I	490	0.10	—	—	—	—
FITC II	490	0.11	—	—	—	—
Normal mouse IgG						
FITC I	494	0.41	1.81	0.71	0.36	—
FITC II	494	0.32	1.23	0.82	0.41	—
4-4-20						
FITC I (1x)¶	506	0.26	0.84	0.98	0.49	508
FITC II (1x)¶	502	0.08	0.91	0.27	0.13	506
FITC I (100x)¶	504	0.56	0.84	2.08	0.01	508
FITC II (0.1x)¶	506	0.04	0.81	0.15	0.77	508

*After subtraction of contribution by FITC (pH 8.3). ‡The number of FITC molecules per antibody. §Binding efficiency. ‖Determined by spectral subtraction of normal mouse IgG labeled with FITC. The same values were obtained for spectral subtraction of FITC free in solution. ¶Denotes molar equivalent of FITC reacted with the antibody.

the presence of a molar equivalent of FITC I (referred to as 1x) based on the number of antibody active sites and possessed a λ_{MAX} of 506 nm. This was close to the maximum shift expected and indicated that essentially all FITC I was bound within the active site. Labeling efficiency for the reaction was 49%. Mab 4-4-20 labeled 1x with FITC II in a parallel reaction had a λ_{MAX} of 502 nm and a labeling efficiency of 13%.

In assessing effects of FITC binding outside the active site on the absorbance spectrum, Mab 4-4-20 was reacted with a large excess of FITC I (100x molar equivalents of active sites). After extensive dialysis, the resulting absorbance spectrum contained a single averaged λ_{MAX} at 504 nm. This indicated that the λ_{MAX} of 502 nm obtained for 4-4-20 labeled with FITC II represented an average value.

To determine a more accurate value of λ_{MAX} for FITC II within the active site, binding outside the active site was minimized by labeling 4-4-20 with 1/10 a molar equivalent of FITC II. The λ_{MAX} for this sample was 506 nm, the same as that obtained for 4-4-20 labeled with a molar equivalent of FITC I. Thus, all FITC II was bound within the active site and there were no differences in the spectral shifts for FITC I and II when bound within the 4-4-20 active site. Therefore, the λ_{MAX} of 502 nm obtained for 4-4-20 labeled 1x with FITC II was the result of significant binding external to the active site.

To confirm that the λ_{MAX} of FITC II within the 4-4-20 active site was the same as FITC I, spectral subtractions were performed. Because the percentages of FITC within and outside the active site were not known, a relatively simple approach was taken. Absorbance of FITC bound to 4-4-20 at 494 or 496 nm (for FITC I and II, respectively) was matched with the absorbance of FITC I or II bound to normal mouse IgG at 494 or 496 nm. The absorbance spectrum charactizing FITC bound to normal mouse IgG was subtracted from the appropriate spectrum of 4-4-20 labeled with FITC. The same method was used to subtract the spectra of free FITC I or II from the spectrum of 4-4-20 labeled with FITC. In both cases, the resultant spectra showed a λ_{MAX} of 508 nm for 4-4-20 labeled 1x and 100x with FITC I and 0.1x with FITC II. The λ_{MAX} for 4-4-20 labeled 1x with FITC II was 506 nm. Because resolution in this region was ±2 nm, subtraction of contributions from FITC bound outside the active site of 4-4-20 resulted in the same spectral shift in all cases (508 ± 2 nm). This provided further evidence that there were no detectable differences in the environment of FITC I and II within the 4-4-20 active site.

Absorbance data indicated that although there was a 100-fold difference in the amount of FITC I used to label Mab 4-4-20 in the two labeling experiments (1x and 100x), there was only a twofold increase in the amount of FITC I bound to 4-4-20. Because the spectral shift was only 2 nm less than that obtained for 4-4-20 labeled 1x with FITC I, and the *r* value was only slightly greater than two (all active sites liganded) it appeared that little FITC I was located outside the active site. Because labeling efficiency for normal mouse IgG was ~40%, this suggested that upon FITC I binding within the active site additional binding of FITC outside the active site became increasingly difficult. This may result from domain tightening in the antibody upon FITC binding (20). Failure to label outside the active site may also correlate with the relative rates of hydrolysis of the label (at the concentrations of reagent used) in comparison to the rate at which adducts are formed between FITC and multiple lysine residues.

Association rate analysis

The association rate for FITC I binding with Mab 4-4-20 (data not shown) was determined to be $10.80 \pm 1.13 \times 10^6\ M^{-1}s^{-1}$ (average of five trials) which is a factor of two higher than that obtained by Herron (17). The maximum amount of FITC I bound ranged from 98 to 99%. Association kinetics describing binding of FITC II to 4-4-20 were determined to contain a fast and slow component with rates of $5.01 \pm 0.23 \times 10^3$ and $1.92 \pm 0.04 \times 10^3\ M^{-1}s^{-1}$, respectively. Associations rates for FITC II were the result of three trials, and the maximum amount of FITC II bound was 92.5%.

Since for even the fast component, binding of FITC II by 4-4-20 was more than 2,000 times slower than that observed for FITC I, it appeared that the difference in the position or orientation of the isothiocyanate group must result in significant steric hindrance to binding within the active site of 4-4-20. The latter accounted for the decreased binding of FITC II within the 4-4-20 active site observed in absorbance measurements.

Lifetime measurements

Phase and modulation data for FITC I and II free in solution and covalently bound to N-acetyl-L-lysine, normal mouse IgG, and Mab 4-4-20 were fit to models containing one to three discrete lifetimes using Globals Unlimited (18). FITC I and II displayed a 3.86 and 1.33 ns components free in solution and bound to N-acetyl-L-lysine and normal mouse IgG. When bound within the active site of Mab 4-4-20, FITC I and II were found to have a short-lived component due to fluorescence quenching of FITC within the active site. Chi-square values (χ^2, Table 2), decreased significantly when the number of lifetimes in the model increased from one to two. For some samples, the χ^2 values reported for a two component fit actually represented a three component

TABLE 2 **Global analysis chi-square values for discrete and distributed lifetime models**

Sample	Discrete			Distributed		
	Single exponential	Double exponential	Triple exponential	Lorentzian	Single Gaussian	Single uniform
FITC I	90.0	0.9*	—	1.4	2.2	3.6
FITC II	65.7	2.7*	—	3.2	4.6	5.2
N-Acetyl-L-Lysine						
FITC I	44.7	1.1*	0.9‡	1.4	1.4	2.9
FITC II	53.5	1.3	0.8‡	1.8	2.1	4.3
Normal mouse IgG						
FITC I	130.5	2.1	0.4	4.3	1.8	5.7
FITC II	116.6	0.8	0.9	5.0	1.3	3.6
4-4-20						
FITC I (1x)§	788.4	1.6	1.9	35.5	308.3	457.1
FITC II (1x)§	783.8	1.9*	—	23.3	4.1	7.5
FITC I (10x)§	589.1	0.8	0.9	8.0	162.1	275.4
FITC II (0.1x)§	1006.1	1.4*	—	25.9	205.1	378.1
FITC I (100x)§	213.9	2.1	2.2	24.3	4.1	12.7

*All numbers stated are chi-square values. A three component fit in which the third component had a long lifetime and was derived from less than one percent of the fluorescent species. ‡This fit gave a small percentage of a long-lived component. §Denotes molar equivalent of FITC reacted with the antibody.

fit in which the third component had a long lifetime and was derived from <0.5% of the species (Table 2). Because the fluorescence lifetime was relatively long and the amount of that species present small, the third component was thought to be either a contaminant or an instrumental artifact not directly attributable to FITC. Therefore, a two component fit was considered appropriate.

To determine whether the fluorophore population contained two discrete lifetimes or a single distributed lifetime, data were fit to models for Lorentzian, Gaussian, and uniform distributed lifetimes (Table 2). FITC I and II free in solution and covalently bound to N-acetyl-L-lysine and normal mouse IgG, fit two discrete lifetimes only slightly better than for a single Lorentzian or Gaussian distribution but significantly better than in an uniform distribution. However, for Mab 4-4-20 affinity labeled with FITC, two discrete lifetimes fit the data 2–30 times better than the best fit obtained for a distributed lifetime. Because two discrete lifetimes were found to best fit the data, all further analyses were performed using the two discrete lifetimes model.

Lifetime resolved fluorescence quenching

Quenching studies were performed on samples listed in Table 2 as a means of assessing ligand (FITC) accessibility to iodide in each sample as well as a means of providing data sets to establish confidence intervals. For each sample, phase and modulation measurements were taken from 2 to 150 MHz for 5–6 different quencher concentrations (ranging from 0–0.3 M KI). Data sets were analyzed using Globals Unlimited and fit to two discrete lifetimes and two quenching constants and for comparison with one lifetime and one quenching constant. For each data set, lifetime and quenching constants were linked and the mole fractions varied. All results and confidence intervals obtained are summarized in Table 3. The data were clearly best fit by a two component, two quenching constant model.

Error analysis

Confidence intervals reported in Table 3 were determined by performing error analyses on data sets using Globals Unlimited. A complete minimization was performed for each fixed value of the tested parameter. Errors were calculated using the maximum allowable error in phase (±0.20) and modulation (±0.01). Confidence intervals reflect the minimum and maximum values of the lifetime or quenching constant for which χ^2 falls below the 67% confidence interval (1 SD).

Fluorescence lifetimes

FITC I and II free in solution were both found to possess 3.86 ± 0.16 and 1.33 ± 0.24 ns lifetime components (Table 3). Both lifetimes were obtained for FITC covalently bound to N-acetyl-L-lysine and normal mouse

TABLE 3 **Global analysis of phase and modulation lifetime and quenching data**

	Two discrete exponential lifetimes and two quenching constants							One r, One K
Sample	f_1*	τ^1 (ns)	K_1 (M^{-1})	f_2*	τ^2 (ns)	K_2 (M^{-1})	χ^2	χ^2
FITC I	0.66	3.86 ± 0.16	2.30 ± 0.22	0.34	1.33 ± 0.24	3.56 ± 2.17	0.62	48.58
FITC II	0.66	3.53 ± 0.34	2.25 ± 1.79	0.34	1.18 ± 0.71	3.47 ± 3.35	1.58	51.59
N-Acetyl-L-Lysine								
FITC I	0.75	4.14 ± 0.31	2.18 ± 0.34	0.25	1.74 ± 0.46	3.02 ± 1.81	1.42	20.70
FITC II	0.73	3.97 ± 0.18	2.07 ± 0.23	0.27	1.32 ± 0.35	2.35 ± 2.44	1.08	25.48
Normal mouse IgG								
FITC I	0.59	3.89 ± 0.22	0.56 ± 0.11	0.41	1.38 ± 0.23	1.66 ± 0.76	2.71	116.28
FITC II	0.59	3.89 ± 0.14	0.57 ± 0.11	0.41	1.28 ± 0.16	1.72 ± 1.06	1.56	130.53
4-4-20								
FITC I (1x)	0.04	3.89 ± 0.32	2.08 ± 0.39	0.96	0.37 ± 0.03	−3.01 ± 3.40	1.86	3877.85
FITC II (1x)	0.35	3.34 ± 0.14	0.56 ± 0.07	0.65	0.67 ± 0.08	2.34 ± 0.91	2.28	460.11
FITC I (10x)	0.06	3.72 ± 0.42	3.05 ± 0.39	0.94	0.44 ± 0.05	0.68 ± 1.51	2.33	114.35
FITC II (0.1x)	0.10	3.82 ± 0.23	0.69 ± 0.25	0.90	0.49 ± 0.04	1.13 ± 1.71	1.66	624.98
FITC I (100x)	0.13	2.97 ± 0.32	0.38 ± 0.33	0.87	0.74 ± 0.06	0.77 ± 0.68	2.23	116.03

*Mole fraction (not fractional intensity).

immunoglobulin molecules. Because a normal mouse antibody population lacks detectable specific antifluorescein activity, FITC is most likely bound to lysine residues external to the immunoglobulin active site. Invariance in fluorescence lifetime values demonstrated that neither covalent binding of the FITC molecule nor interactions of the FITC molecule with residues outside the antibody active site molecule affect the FITC lifetime. The percentage of the 3.86 and 1.33 ns components was the same for FITC I and II and varied only ±10% when bound to N-acetyl-L-lysine or normal mouse IgG. Thus, there was no indication of preferential binding of one component relative to another. The two lifetime components (3.86 and 1.33 ns, Table 3) appeared to be primarily due to pH 8.3, which was employed based on the antibody binding experiments. In 0.1 N NaOH, fluorescein and FITC (I and II) in the dianionic form showed almost exclusively the 3.86 ns component. Thus, at pH 8.3 it appears that the monoanionic form of fluorescein correlates with the 1.33 ns component.

For Mab 4-4-20 labeled with a molar equivalent (1x) of FITC I, 96% of the FITC molecules were found to have a lifetime of 0.37 ns and 4% to have a lifetime of 3.89 ns. Absorbance measurements indicated that essentially all FITC was bound within the active site. Kinetic studies showed that FITC I was bound to the active site within 15 s at 2°C. Therefore, FITC I was apparently "pocketed" by the Mab 4-4-20 before it bound outside the active site. Thus, the 0.37 ns lifetime reflects ligand within the active site. Quenching of fluorescein bound within the active site of 4-4-20 has ranged between 90 and 95% (7). It has been suggested that tryptophan, and in one case histidine residues, are involved in antibody active site quenching of bound fluorescein (5). X-Ray crystallographic results revealed that the xanthonyl ring of fluorescein fits a narrow slot in the 4-4-20 active site, the bottom of which is formed by tryptophan L101 and the sides by tryptophan H33 and tyrosine L37 (10). Such interactions may result in a decreased fluorescence lifetime of fluorescein within the 4-4-20 site. A decrease in the lifetime of fluorescein from 3.86 ns outside the active site to 0.37 ns within the active site is proportional to fluorescence quenching within the active site. This suggested that 0.37 ns was a reasonable active site lifetime for FITC and the observed quenching was predominantly dynamic. Of the remaining FITC 4% had a lifetime of 3.86 ns which corresponds to FITC either free in solution or bound to lysine residues external to the antibody active site.

When Mab 4-4-20 was labeled with a molar equivalent (1x) of FITC II, only 65% of the molecules were found to exhibit a short lifetime. Because only 13% of FITC II added bound to 4-4-20, this corresponded to 9% of the FITC II binding within the 4-4-20 active site. Decreased amounts of FITC II bound within the active site most likely reflected the slow association rate for binding with 4-4-20, permitting extensive external binding. The fluorescence lifetime of the short component of 4-4-20 labeled 1x with FITC II was nearly twice as long as the lifetime of FITC I within the active site. In addition, the lifetime of the species outside the active site was 3.34 ± 0.14 ns, which was shorter than that observed for FITC bound to normal mouse IgG (3.89 ± 0.14 ns). Because normal mouse IgG labeled with FITC gave two lifetimes,

3.89 and 1.38 ns, one would expect that 4-4-20 labeled with FITC would have displayed three lifetimes: a 3.89 and a 1.38 ns component corresponding respectively to FITC bound outside of the active site, and a short lived component corresponding to FITC within the active site.

Data simulations

Because none of the FITC-labeled Mab 4-4-20 samples fit the three component model, simulations were performed to determine resolvability of the three lifetimes. All simulated data were analyzed over the frequency range of 1–160 MHz and 1 MHz–1 GHz. In the first simulation, the data were analyzed over a frequency range of 1–160 GHz, and χ^2 for the three component fit was not significantly better than χ^2 for a two component fit. This confirmed that the data were best fit by a two component model.

In the second simulation, which represented Mab 4-4-20 labeled 1x with FITC II, similar trends were evident. Simulated data analyzed over a range of 1–160 MHz in a three component fit gave only a slightly better χ^2 than the two component fit. In the two component fit, the long component was determined to have a shorter lifetime and the short component to have a significantly longer lifetime than the simulated value. This was consistent with data obtained characterizing Mab 4-4-20 labeled 1x with FITC II. Thus, the 4-4-20 data could have been the result of three components with lifetimes and mole fractions similar to those used in the generation of the simulated data.

Based on the simulation studies, the two lifetimes model for FITC (I and II) labeled Mab 4-4-20 would be consistent with active site amino acid-ligand interactions revealed by high-resolution crystallographic studies (10). The two pKa values for free fluorescein are 6.7 and 4.4, whereas the two values in the bound state are lower. Thus, the 1.33 ns lifetime indicative of a monoanionic subspecies of free fluorescein may not exist in the antibody bound state due to conversion of the 3.86 and 1.33 ns components to a quenched 0.37 ns species. This suggests that all antibody bound ligand molecules are in the same ionic state.

Iodide quenching constants

Phase and modulation data, as a function of quencher concentration, are shown in Fig. 1 *a* for normal mouse IgG labeled with FITC I (1x) and in Fig. 1 *b* for 4-4-20 labeled with FITC I (1x). In the case of normal mouse IgG, FITC I was covalently attached to lysine residues at various positions outside the active site. A shift in the phase and modulation curves to higher frequencies for FITC bound to normal mouse IgG with increasing

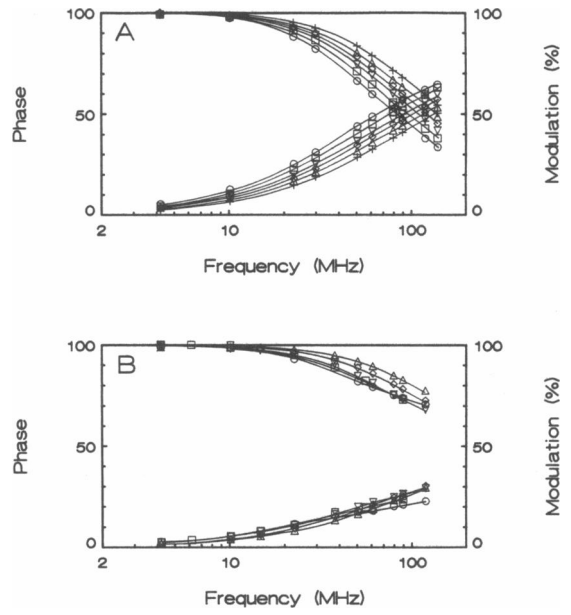

FIGURE 1 Phase and modulation data as a function of quencher concentration for FITC I bound to (*A*) normal mouse IgG and (*B*) 4-4-20. In each case the lower curves correspond to values of phase and the upper curves to values of modulation. Symbols are as follows: (*A*) [KI], in molar: 0, (○); 0.05, (□); 0.10, (▽); 0.15, (◇); 0.20, (△); 0.30, (+); (*B*) [KI], in molar: 0, (○); 0.025, (□); 0.05, (▽); 0.10, (◇); 0.15, (△).

quencher concentration (Fig. 1 *a*) showed the accessibility of FITC to iodide. Fig. 1 *b* shows only a slight shift to higher frequencies as a function of quencher concentration. This indicated that FITC within the active site of 4-4-20 was inaccessible to iodide consistent with previous findings (7).

Quenching constants (K_1 and K_2) are summarized in Table 3. No difference in quenching was found between FITC isomers I and II, and between FITC free in solution or bound to *N*-acetyl-L-lysine. Thus, quenching of FITC by iodide was unaffected by linkage to lysine. FITC bound to normal mouse IgG was found to have a smaller quenching constant than FITC free in solution. Decreased solvent accessibility may have been due to FITC binding to nonspecific grooves or pockets on the surface of the antibody molecule partially shielding it from solvent. Because the quenching constant for FITC I within the 4-4-20 active site was essentially zero, it implied that iodide was excluded. The latter was consistent with the tight fit of fluorescein within the binding pocket of 4-4-20 as determined by crystallographic data (10). The quenching constant for FITC I outside the active site corresponded to the quenching constant for free FITC rather than FITC bound to normal mouse IgG.

Mab 4-4-20 labeled 1x with FITC II had a relatively

large quenching constant for the short lived component. Assuming that the quenching constant contained a contribution from the species within the active site plus a contribution from the 1.33 ns component, then it might be expected that the value of the quenching constant would be intermediate between 0 and 1.7 M^{-1}. However, the quenching constant was 2.3 M^{-1} which was larger than the quenching constant obtained for either the 1.33 ns or the 3.86 ns component of normal mouse IgG. For FITC II labeled 0.1x with FITC II, the quenching constant for the 3.86 ns component was the same as that obtained for normal mouse IgG.

Stern-Volmer analysis

Because iodide usually quenches primarily by promotion of intersystem crossing to the triplet state and subsequent quenching by other processes, it was anticipated that quenching of FITC by iodide would be predominantly dynamic in nature. To determine static or dynamic quenching, or a combination of both, plots of the fraction of fluorescence intensity remaining (F_o/F) and the fractional average lifetime ($\langle\tau_o\rangle/\langle\tau\rangle$) were made according to the Stern-Volmer relationship,

$$F_o/F = 1 + k_q\tau_o[Q] = 1 + K_{sv}[Q], \quad (3)$$

where k_q is the bimolecular quenching constant and K_{SV} the Stern-Volmer quenching constant. Because K_{SV} contains contributions from both static (K_S) and dynamic (K_d) quenching, the slope of a plot of F_o/F vs. $[Q]$ is K_{SV}, where $K_{SV} = K_S - K_d$. Because the fractional average lifetime is related to the quencher concentration by the equation

$$\langle\tau_o\rangle/\langle\tau\rangle = 1 + K_d[Q], \quad (4)$$

the slope of a plot of $\langle\tau_o\rangle/\langle\tau\rangle$ vs. $[Q]$ gives K_d, the dynamic contribution to the quenching constant. Static contributions to the quenching constant are determined by taking the difference in the slopes of the plots of F_o/F and $\langle\tau_o\rangle/\langle\tau\rangle$ vs. $[Q]$. Slopes (Table 4) of the plots of F_o/F and $\langle\tau_o\rangle$ vs. $[Q]$ showed that quenching of FITC by iodide was almost entirely dynamic.

The quenching constants followed similar trends as those obtained for Global analysis of phase and modulation data. Quenching constants measured for FITC bound to normal mouse IgG were found to be smaller than those obtained for FITC free in solution or bound to *N*-acetyl-L-lysine. Plots of F_o/F and $\langle\tau_o\rangle/\langle\tau\rangle$ vs. $[Q]$ are shown in Fig. 2 *a* for FITC I, and in Fig. 2 *b* for FITC II bound to normal mouse IgG and 4-4-20.

The difference in quenching of FITC bound outside the antibody active site relative to within the active site of a specific antibody is illustrated in Fig. 2 *a*. Quenching

TABLE 4 Quenching constants derived from Stern-Volmer analyses

Sample	K_{sv}*	K_d‡	K_s§
FITC I	9.27 ± 0.97	9.12 ± 0.46	0.63
FITC II	9.71 ± 0.52	9.01 ± 0.31	0.69
N-Acetyl-L-Lysine			
FITC I	10.07 ± 0.64	9.75 ± 0.37	0.32
FITC II	9.27 ± 0.59	8.74 ± 0.14	0.53
Normal mouse IgG			
FITC I	3.79 ± 0.39	3.10 ± 0.19	0.69
FITC II	3.33 ± 0.38	2.72 ± 0.08	0.61
4-4-20			
FITC I (1x)‖	0.80 ± 3.41	0.55 ± 2.45	0.25
FITC II (1x)‖	1.82 ± 0.16	1.88 ± 0.13	−0.07
FITC I (10x)‖	0.69 ± 0.02	2.01 ± 0.16	−1.32
FITC II (0.1x)‖	0.35 ± 0.75	2.15 ± 0.54	−1.80
FITC I (100x)‖	1.59 ± 0.28	1.31 ± 0.40	0.28

*Stern-Volmer quenching constant derived from the slope of F_o/F vs. $[Q]$. ‡Dynamic quenching constant from the slope of $\langle\tau_o\rangle/\langle\tau\rangle$ vs. $[Q]$. §Static quenching constant, calculated from $K_s = K_{sc} - K_d$. ‖Denotes molar equivalent FITC reacted with Mab 4-4-20.

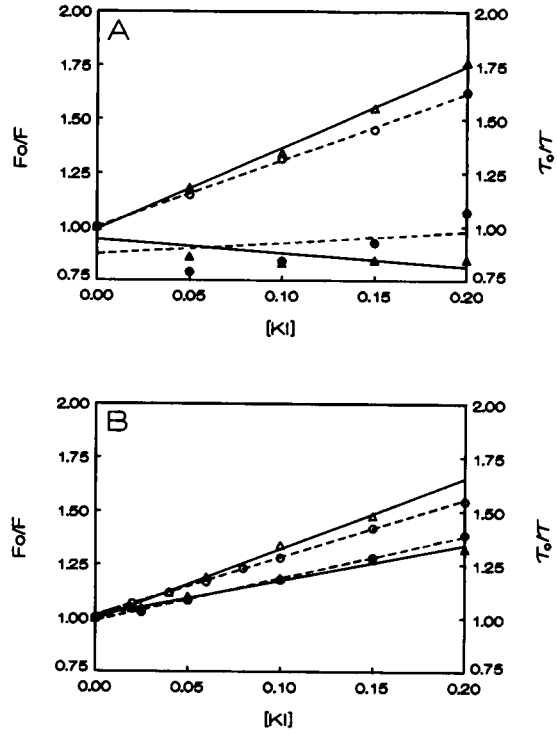

FIGURE 2 F_o/F vs. [KI], (in molar), for NMIgG (—△—) and 4-4-20 (—▲—) and $\langle\tau_o\rangle/\langle\tau\rangle$ vs. [KI], (in molar), for NMIgG (-○-) and 4-4-20 (-●-). Samples are labeled 1x with (*A*) FITC I and (*B*) FITC II.

constants for FITC I bound to normal mouse IgG were 3.79 and 3.10 M^{-1} for K_{SV} and K_d, respectively. These values represented quenching constants for FITC bound outside the antibody active site. For 4-4-20 labeled 1x with FITC I, it had been established that essentially all (96%) FITC was bound within the active site. As can be seen in Fig. 2 *a*, the quenching constants obtained were small (K_{SV} = 0.80 M^{-1} and K_d = 0.55 M^{-1}). Thus, FITC within the active site was considerably less accessible to iodide than FITC bound outside of the active site.

As shown in Fig. 2 *b*, Mab 4-4-20 labeled 1x with FITC II had quenching constants intermediate between those found for normal mouse IgG and 4-4-20 labeled 1x with FITC I. For 4-4-20 labeled 1x with FITC II, 35% of the FITC was bound outside of the active site and 65% within the active site. The quenching constants obtained were 1.82 and 1.88 M^{-1} for K_{SV} and K_d, respectively. These values were about twice those expected assuming a linear relationship between the percent of FITC bound outside of the active site and the quenching constant (K_{SV} = 1.09 M^{-1}, K_d = 0.93 M^{-1}).

Consideration of these data in the context of results obtained from Global analysis of lifetime resolved quenching measurements, indicated that the quenching constant for FITC II within the active site of 4-4-20 was significantly greater than the constant for FITC I within the active site. Significant accessibility of FITC II to iodide when bound within the 4-4-20 active site suggested that the active site existed at least on an average, in a more open configuration. The latter could be the result of steric constraints, which either force the active site into a more open conformation or result in a broader spectrum of conformational fluctuations of the active site (relative to binding of FITC I) in an effort to accommodate the FITC II isomer (20).

CONCLUSION

Suitability of the antifluorescein antibody model is directly dependent on advantageous spectral properties of the homologous fluorescein ligand. In particular Mab 4-4-20 has served as a high affinity prototype. In this report, the active site microenvironment of 4-4-20 has been studied comparatively when bound to the homologous ligand and to an isomeric hapten (FITC I and II, respectively). Results show that the homologous ligand (isomer I) has an association rate >2,000 times that of the isomeric ligand II, probably due to favorable steric considerations. Once within the active site both isomers exhibit the same fluorescence lifetimes and degree of spectral shift, indicating almost identical microenvironments. However, when the isomeric ligand is bound it is significantly more accessible to solvent than the homologous ligand, suggesting that 4-4-20 may exist in a more open configuration.

Based on the three-dimensional crystallographic structure of the 4-4-20 active site, the planar xanthonyl moiety of fluorescein fits tightly into a slot near the bottom of the site (10). Because FITC I is the homologous ligand and gives identical spectral and fluorescence properties when bound to 4-4-20, it is reasonable to assume that the isothiocyanate derivative of fluorescein fits into the same active site slot. The xanthonyl moiety is aromatic, planar, highly delocalized, and based on a favorable alignment with the phenylcarboxyl group plays a significant role in ligand fluorescence properties. Because fluorescence lifetimes and spectral shifts were the same for both isomers, it is likely that the environment surrounding the xanthonyl moiety is the same for both isomers when bound within the 4-4-20 active site. Therefore, although isomeric ligand (II) diffuses more slowly into the active site, probably due to the sixth-position of the isothiocyanate group, once bound, the xanthonyl portion of the ligand fits into the same slot as the homologous ligand (I).

Based on the resolved structure of the liganded 4-4-20 active site, the phenylcarboxyl and xanthonyl groups constituting fluorescein occupy specific subsites (10). With FITC I bound to the active site the fifth-position on the phenyl carboxyl group is accommodated through a spacing that leads to the aqueous phase surrounding the variable domains. It is apparent that if the isothiocyanate group occupies the fifth-position it is favorably positioned for covalent conjugation with the ϵ-amino group of lysine H52b (10). However in the case of FITC II the isothiocyanate group in the sixth-position is sterically inhibited when bound to the 4-4-20 site. The latter relates to one of the important features of the crystal structure of 4-4-20, namely that two residues (Tyr H53 and Asn L28) form a "lid" over the bound fluorescyl ligand. It would appear that these two residues assume their position as a consequence of ligand binding. Conceivably bound FITC (II) may inhibit formation of the "lid" by residues Tyr H53 and Asn L28 consistent with the observed iodide accessibility. Establishment of the "lid" with bound FITC (I) would be consistent with iodide inaccessibility. Specific formation and location of the covalent bond upon affinity labeling with FITC (II) is unclear at this point.

It is now generally accepted that instead of being rigid, proteins are flexible molecules which dynamically fluctuate between slightly different conformational substates (21, 22). The existence of variable region conformational substates in antibodies (20) seems a reasonable extension of this premise and impacts important immu-

nological phenomena such as affinity and cross-reactivity. Bound homologous ligand stabilizes a particular conformer whereas a cross-reacting ligand may stabilize a different conformer due to structural differences. Results presented in this manuscript based on the reactivity of a homogeneous antibody with two isomers of a hapten are consistent with active site configurational changes and adaptations to accommodate structural differences in the antigen.

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