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Proximity of Antibody Binding Sites Studied by Fluorescence Energy Transfer†

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ABSTRACT: Fluorescence energy transfer experiments by steady-state and nanosecond monophoton techniques were carried out with a covalently linked hybrid rabbit IgG antibody containing one antilactose site and one anti-Dns [5-(dimethylamino)-1-naphthalenesulfonyl] site. The hybrid antibody was prepared from antilactose and anti-Dns antibody by mild reduction, dissociation into half-molecules in acid, and random reassociation with re-formation, to the extent of 80%, of the single disulfide bond between the heavy chains. Fractionation with an antilactose-specific immunoadsorbent yielded a population in which each IgG molecule contained no more than one anti-Dns site per antibody. The acceptor molecules used for intramolecular energy transfer were derivatives of *p*-aminophenyl β-lactoside (PAPL): (dimethylamino)benzeneazo-PAPL and *N*-fluorescyl-PAPL. The fluorescence lifetime (24 ns) and quantum yield (0.57) of the bound Dns group were unaffected by the presence of the acceptor in the adjacent site. Three models were used to calculate the minimum distance between the adjacent sites of the IgG antibody based on the overlap in the emission and absorption spectra of the donor–acceptor pairs and the segmental flexibility of the immunoglobulin molecule. The calculations yielded values in the range of 5.5–7.0 nm for the minimum distance of separation between the antibody sites in solution and demonstrated a substantial energy barrier to the closer approach of the sites.

The property of segmental flexibility inherent and distinctive to the immunoglobulin molecule has been established by sedimentation velocity (Noelken et al., 1965), by electron microscopy (Valentine & Green, 1967; Schumaker et al., 1979), and by time-dependent fluorescence polarization using monophoton technology (Yguerabide et al., 1970; Chan & Cathou, 1977; Lovejoy et al., 1977). Such molecular flexibility undoubtedly plays an important role in the ability of antibody to establish multivalent attachment when identical reiterated antigenic determinants are presented, as in the cases of viral, bacterial, and neoplastic surface antigens. These multiple interactions result in enhancement of intrinsic affinities, thereby increasing the efficiency of immunological responses. In addition, it may be that segmental flexibility plays a role in the regulation of complement activation by immunoglobulin in which diminution of flexibility due to antigen binding allows for more efficient recognition of the Clq binding site of the $C_\gamma 2$ domain.

In an ingenious attempt to define the limits of molecular flexibility and to delineate the overall shape of IgG in solution, Werner et al. (1972) attempted to determine the average distance between the two antigen combining sites of IgG by measuring the efficiency of fluorescent energy transfer between a Dns[1] donor and a fluorescein acceptor positioned in the

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respective combining sites of rabbit hybrid anti-Dns/anti-fluorescyl IgG. Since no energy transfer was observed, the authors concluded that the minimum distance of approach for the adjacent sites was no less than 90 Å, based upon the spectral properties of the donor–acceptor pair used in the experiment.

An ambiguity in the interpretation of these results has become apparent, however, with the recent X-ray crystallographic analysis at 4-Å resolution of the Fc portion of the immunoglobulin molecule (Deisenhofer et al., 1976a,b). This study demonstrated the lack of noncovalent interactions between the two $C_\gamma2$ domains due to the intervention of the carbohydrate chains. In the energy transfer experiment the authors followed the classic procedure for the formation of rabbit hybrid IgG involving alkylation of reduced interchain disulfide bonds. As a result, the single inter-heavy-chain disulfide linkage was irreversibly eliminated. The only remaining interactions which held the hybrid IgG in a four-chain unit were the noncovalent ones between the $C_\gamma3$ domains. This modification could have resulted in an artificial extension of the native molecular configuration with the distance between the combining sites increased by as much as 80 Å due to the separation of the $C_\gamma2$ domains.

In addition to the X-ray crystallographic data, there are several biochemical and biophysical observations which support the hypothesis that the $C_\gamma2$ domains are not involved in the noncovalent association of the half-molecules in reduced and alkylated IgG. First, despite the failure to detect conformational changes with reduced and alkylated IgG compared to the native molecule by using circular dichroism (CD) and optical rotatory dispersion (ORD) (Bjork & Tanford, 1971; Stevenson & Dorrington, 1970; Azuma et al., 1974), a conformational change was inferred from hydrogen–deuterium exchange experiments (Venyaminov et al., 1976). These experiments led to the conclusion that for human IgG the splitting of interchain disulfide bonds resulted in an increase in conformational mobility with a decrease in the free energy barrier to solvent exposure. Second, a comparison of nanosecond fluorescence polarization of Dns-lysine bound to rabbit anti-Dns IgG in the native and in the reduced and alkylated state demonstrated a greater rotational freedom for the chemically modified protein (Chan & Cathou, 1977). Third, characterization of $C_\gamma2$ and $C_\gamma3$ domains from human IgG obtained by cleavage of acid-treated Fc fragments with trypsin demonstrated that the isolated $C_\gamma3$ was in a noncovalent dimeric form. The $C_\gamma2$ fragment was obtained as a covalently linked dimer which, upon mild reduction and alkylation, dissociated into monomers (Ellerson et al., 1976). Finally, Facb fragments of rabbit IgG, which lack the $C_\gamma3$ domain, were mildly reduced and alkylated. The generation of a single component with a molecular weight of 54 000 indicated that the splitting of the interchain disulfide resulted in dissociation into half-molecules (Michaelsen et al., 1975).

The implication of these experiments is that in the complete IgG molecule there are two principal structures which hold the Fc portions, hence the H chains, in close proximity: the inter-heavy-chain disulfide(s) adjacent to the $C_\gamma2$ domains and the noncovalent interactions between the two $C_\gamma3$ domains. Upon reduction and alkylation of interchain disulfides, the Fab portions of IgG remain conformationally intact due to the strong noncovalent interaction between pairs of domains. In the Fc portion, the tertiary protein conformation remains essentially intact, but only the noncovalent one-domain bonding of the $C_\gamma3$'s holds the heavy chains together. The lack of interaction between the two $C_\gamma2$ domains imparts additional modes of flexibility to the reduced IgG molecule beyond those associated with the Fab arms.

Unequivocal interpretation of the energy-transfer experiment with donor–acceptor pairs located in adjacent hybrid IgG combining sites requires an IgG population in which the inter-heavy-chain disulfide bond is retained. The system described in this report utilizes covalently hybridized rabbit anti-Dns/antilactosyl antibody in which complete reoxidation of the light–heavy chain interchain disulfides and 80% reoxidation of the inter-heavy-chain disulfides were achieved. The donor–acceptor pair for these experiments is N^ϵ-Dns-lysine bound to the anti-Dns site and either *p*-[*p*-(dimethylamino)-benzeneazo]phenyl β-lactoside (Lac-Dye) or a fluorescyl derivative of *p*-aminophenyl β-lactoside (Fls-PAPL) bound to the antilactosyl site. There are several advantages associated with the antilactose antibody system for this type of experiment: (1) the capability of eluting both antilactose antibody and anti-Dns/antilactosyl hybrids from Lac-Sepharose immunoadsorbents with lactose, rather than denaturing conditions, and complete removal of the eluting hapten by dialysis; (2) the option of using several different types of energy acceptors for the same hybrid preparation by synthesis of the appropriate lactosyl derivatives; (3) the use of an acceptor chromophore which possesses rotational mobility beyond that of the segmentally flexible molecule, thereby ensuring the randomness of its orientation; (4) the potential for competitive elution of the acceptor from its position at the antilactosyl combining site with the nonchromophoric ligand lactose. The results are analyzed and discussed in terms of the dynamics of a flexible molecular model of IgG.

Materials and Methods

Immunization and Antibody Purification. Proteins derivatized with the 5-(dimethylamino)naphthalene-1-sulfonyl (Dns) fluorophore for immunization and purification of specific antibody were generated as described by Weber (1952). The protein solution (20 mL containing 5 mg/mL 0.1 M sodium bicarbonate, pH 9.5) was treated with 10–30 mg of Dns-Cl in 1 mL of acetone. The reaction was allowed to proceed overnight in the dark at 4 °C. After centrifugation, the supernatant was fractionated by gel filtration with a Bio-Gel P-6 column in 0.1 M NH_4HCO_3, and the void volume peak was pooled and lyophilized. For the preparation of the immunogen Dns-hemocyanin (Dns-Hy), a ratio of *Limulus polyphemus* hemolymph Type VI to Dns-Cl of 100:30 (w/w) was used. The number of moles of Dns per 10^5 g of hemocyanin was 18 as measured by optical density with $E_{cm}^{1\%} = 11.2$ for hemocyanin at 280 nm and molar absorbances of 4.57×10^3 and 3.03×10^3 for the Dns group at 330 and 280 nm, respectively. For the preparation of the immunoadsorbent, bovine serum albumin (BSA) (Sigma Chemical Co.) was reacted with Dns-Cl at a weight ratio of 100:12, resulting in Dns_{13}-BSA, assuming a molecular weight of 6.6×10^4 and $E_{cm}^{1\%} = 6.67$ for the BSA at 280 nm. The Dns-BSA was covalently coupled to stabilized CNBr-activated Sepharose 4B (Pharmacia) by following the manufacturer's recommended procedure.

[1] Abbreviations used: PBS, 0.01 M phosphate buffer, 0.85% NaCl with 0.01% NaN_3, pH 7.2; Dns, 5-(dimethylamino)-1-naphthalenesulfonyl; Dns-lysine, N^ϵ-Dns-L-lysine; PAPL, *p*-aminophenyl β-lactoside; Fls-PAPL, *N*-fluorescyl-*p*-aminophenyl β-lactoside; Dns-PAPL, *N*-Dns-*p*-aminophenyl β-lactoside; pyrene-PAPL, *N*-(*N*-succinylaminopyrenyl)-*p*-aminophenyl β-lactoside; fluoranthenyl-PAPL, *N*-(*N*-succinylaminofluoranthenyl)-*p*-aminophenyl β-lactoside; Lac-Dye, *p*-[*p*-(dimethylamino)benzeneazo]phenyl β-lactoside; Hy, *Limulus polyphemus* hemolymph Type VI; BSA, bovine serum albumin; BGG, bovine γ-globulin; $NaDodSO_4$, sodium dodecyl sulfate.

The anti-Dns antibody was elicited in white New Zealand female rabbits by monthly injections of 1.0 mL of an emulsion containing 50% CFA (Difco) and 1.5 mg of Dns_{18}-Hy. The immunogen was equally distributed among several intramuscular injections in each thigh and a subcutaneous injection in the neck. The antiserum used for these experiments consisted of a pool of four bleedings from a rabbit 1 week after the secondary set of injections. Three precipitations with 50% saturated ammonium sulfate were made with the antiserum (120 mL), and the final precipitate was dissolved in 0.01 M phosphate buffer, pH 7.2, 0.85% NaCl, and 0.01% NaN_3 (PBS) to a total volume of 80 mL. This solution was dialyzed vs. 1 L of PBS at 4 °C with two changes. The solution was then incubated with a 3-mL packed volume of Dns-BSA–Sepharose 4B which had been washed twice with 25 mL of 1.0 M propionic acid and 4.0 M urea and then neutralized by repeatedly washing with PBS. The incubation was done at 4 °C for 18 h with gentle stirring. After the adsorbent was washed 5 times with 30 mL of PBS each time, the anti-Dns antibody was eluted by two consecutive 30-min incubations with 5.0 mL of 1.0 M propionic acid and 4.0 M urea. The eluted protein solution was brought to pH 5.5 by immediate addition of an equal volume of 1.0 M Tris-HCl, pH 8.0. After adjustment to neutrality with 4.0 M NaOH, the solution was dialyzed twice against 1.0 L of PBS at 4 °C. The IgG component was selected by gel filtration with Sephadex G-200 in PBS. Ouchterlony analysis of this preparation showed a single line of fusion with goat antinormal rabbit serum and goat antirabbit IgG (Miles Labs., Inc.). The rabbit anti-Dns IgG solution at 0.5 mg/mL in PBS demonstrated no measurable Dns fluorescence. A solution of 50 μg/mL demonstrated specific anti-Dns activity by the criteria of protein fluorescence quenching and fluorescence enhancement with Dns-lysine analogous to that described by Parker et al. (1967).

Antilactose antibody was elicited in female white New Zealand rabbits immunized with both *Streptococcus faecalis* (strain N) vaccine (Ghose & Karush, 1973), which contains a cell wall diheteroglycan component possessing multiple lactosyl moieties (Pazur et al., 1973), and PAPL diazotized to bovine γ-globulin (BGG), as previously described (Utsumi & Karush, 1964). The antiserum used in these experiments was obtained after the third course, each course consisting of three intravenous injections per week for three consecutive weeks, at 0.5, 1.0, and 2.0 mg of dry weight per injection for each succeeding week. In addition, at the third weekly injection, 0.5 mL of PAPL–BGG at 1 mg/mL in 50% Freund's complete adjuvant was injected intramuscularly. During the fourth week the rabbits were bled 3 times, days 1, 3, and 5, and then rested for 1 month. The pooled antiserum was passed over an immunoadsorbent prepared by the conjugation of *p*-aminophenyl β-lactoside to CNBr-activated Sepharose 4B (Cuatrecasas et al., 1968). The column was washed with PBS and the specific antibody was eluted with 0.2 M lactose. The antibody preparation was first dialyzed against 0.5 M galactose and then vs. PBS. The protein was concentrated by using an Amicon ultrafiltration cell with a PM-10 membrane and then chromatographed on Sephadex G-200 in PBS to select the IgG component. Both the rabbit anti-Dns and antilactose IgG preparations demonstrated ratios of optical density at 280 nm to that at 250 nm greater than 2.5. Concentrations of protein were obtained from the optical density with $E_{cm}^{1\%} = 14.4$ at 280 nm. Rabbit IgG was assumed to have a molecular weight of 150000.

Hyperimmune antifluorescein antiserum treated with dextran sulfate and twice precipitated with saturated ammonium sulfate was a generous gift from Dr. E. W. Voss, Jr. This preparation was passed through Sephadex G-200 in PBS to select the IgG fraction. The concentration of combining sites was determined graphically from a titration in which Fls-PAPL emission was measured as a function of increasing amounts of protein.

Covalent IgG Hybrid Formation. The procedure for the formation of a covalent rabbit IgG hybrid was modeled upon the method devised by Hong & Nisonoff (1965). Purified rabbit antilactose and anti-Dns IgG's in 0.02 M Tris-HCl buffer, pH 8.0, were mixed in a molar ratio of 7:3 (antilactose/anti-Dns). The solution was flushed with N_2 for a minimum of 30 min prior to reduction and then treated with 2-mercaptoethanol (Eastman) at a final concentration of 20 mM for 60 min at room temperature. After adjustment of the pH to 2.5 with 1.2 N HCl, the reduced protein solution was stirred for 60 min. It was then dialyzed overnight vs. 1.0 L of 0.1 M Tris-HCl, pH 8.0, and 0.85% NaCl at 4 °C to allow for the re-formation of interchain disulfides. Finally, samples were dialyzed twice vs. 1.0 L of PBS at 4 °C for 24 h.

The removal of anti-Dns/anti-Dns hybrid IgG was accomplished by passing the hybrid preparations through a Lac–Sepharose affinity column. After the column was washed with PBS, the protein containing a lactose-specific combining site was eluted with 0.2 M lactose in PBS and dialyzed first against 0.5 M galactose and then PBS. This process selects for populations of antilactose/antilactose and anti-Dns/antilactose hybrid IgG.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was run, using a 4.4% acrylamide gel of which 2.85% was *N,N'*-methylenebis(acrylamide), in 0.1 M PO_4 buffer with 0.1% $NaDodSO_4$ at pH 7.1. To 50–100 μL of protein sample was added 25 μL of saturated $NaDodSO_4$. The mixture was placed in a boiling water bath for 1 min to ensure uniform binding of the $NaDodSO_4$ to protein. For H- and L-chain standards, in addition to the saturated $NaDodSO_4$, 25 μL of 2-mercaptoethanol was added to normal rabbit IgG (NRγG) (Miles) and incubated at 100 °C for 5 min (Weber & Osborne, 1975). Samples were run at 10 mA/gel tube until the bromophenol blue tracking dye had traversed 8.0 cm. Gels were fixed with 10% Cl_3AcOH and stained with 0.05% Coomassie Brilliant Blue R-250 (Eastman) in acetic acid–ethanol–H_2O (14:40:100). Destained gels were scanned at 660 nm with a Gilford Model 240 spectrophotometer equipped with a Model 2410S linear scanner.

Protein Radioiodination. Trace radioiodination of rabbit anti-Dns IgG was achieved using the chloramine-T method (Greenwood et al., 1963). Rabbit anti-Dns IgG (18 μg, 0.12×10^{-6} mmol) was labeled with 0.027×10^{-6} mmol of $Na^{125}I$ for 60 s at pH 7.5. After the addition of sodium metabisulfite and cold NaI, the reaction mixture was added to 1.25 mL of rabbit anti-Dns IgG at 1.85 mg/mL and dialyzed vs. 0.02 M Tris-HCl, pH 8.0, to prepare for hybrid formation. On the basis of 100% efficiency of labeling, the specific activity of the [^{125}I]anti-Dns IgG before dilution with cold protein would be 0.625 μCi/μg or 0.22 ^{125}I atom per IgG molecule.

Synthesis of Fluorescyl-PAPL (Fls-PAPL). A reaction mixture of 1.25 mmol of fluorescein isothiocyanate (FITC), isomer I (Sigma), and 1.0 mmol of *p*-aminophenyl β-lactoside (PAPL) in 5.0 mL of redistilled dimethylformamide was allowed to react in the dark at room temperature for 16 h. Ninhydrin spray of TLC's (95% ethanol) demonstrated complete utilization of PAPL. The dimethylformamide was re-

moved by precipitation with 120 mL of cold diethyl ether. The product was washed with 80 mL of ethyl acetate 5 times, dissolved in 4.0 mL of methanol, and reprecipitated with 30 mL of diethyl ether 5 times to remove the unreacted FITC. Finally, the precipitate was washed twice with 60 mL of diethyl ether and dried in vacuo with P_2O_5 at room temperature. TLC's using Eastman 6061 silica gel sheets showed a single fluorescent spot. Anal. Calcd for $C_{39}H_{38}N_2SO_{16}\cdot H_2O$: C, 55.73; H, 4.76; N, 3.33; S, 3.81. Found: C, 55.62; H, 4.98; N, 3.21; S, 3.69.

Fluorescence Quenching. Steady-state fluorescence quenching of purified antibody by ligand was measured by using a Perkin-Elmer Model 512 double-beam fluorescence spectrophotometer in the ratio mode with diffuse plates in the reference chamber. The excitation and emission wavelengths were 295 and 330 nm, respectively, with 10-nm slits and a glass filter to minimize detection of scattered excitation radiation. The measurements were made by continuous titration using a motor-driven syringe with a total of approximately 200 μL of the appropriate ligand delivered into a 3.0-mL solution of antibody in PBS (50–75 μg/mL) at a rate of 10 μL/min with constant stirring and maintained at 22 °C. Control titrations were performed with a purified human IgG_1 meloma protein to control for dilution and attenuation of the fluorescence signal. The maximum quenching (Q_{max}) was obtained by linear regression analysis of the reciprocal of percent quench vs. the reciprocal of ligand concentration ($1/Q$ vs. $1/[\text{ligand}]$) extrapolated to $1/[\text{ligand}] = 0$. Binding parameters were calculated by using the Sips distribution function with computations performed on a Commodore PET 2001 microcomputer.

For the fluorescence quenching of Fls-PAPL, manual titrations were performed with 0.5 mL of 0.83×10^{-6} M Fls-PAPL in PBS with a fluorescence cuvette (2 × 10 mm; Precision Cells, Inc.). A total of 200 μL of either rabbit anti-lactose IgG, rabbit antifluorescein, or a DEAE-purified human IgG(K) myeloma in PBS was added to the ligand solution in 10-μL aliquots by using a Hamilton syringe. Mixing was accomplished by repeated inversion of the cuvette. The wavelengths for excitation and emission were 480 and 510 nm, respectively.

Fluorescence Polarization. Fluorescence polarization experiments were performed using two 105 UV WRMR polarizers (Visual Products Division of 3M Co.) with one positioned in the converging excitation beam and the other positioned in the diverging beam of luminescence. Ten-nanometer slits were used for excitation and emission with wavelengths of 480 and 510 nm, respectively. The fluorometer was set in the ratio mode and the temperature (20 ± 0.2 °C) was monitored with a Bat-8C digital thermometer (Bailey Instrument Co., Inc.). Antibody solutions were introduced manually with a Hamilton syringe into 0.5 mL of 0.92×10^{-6} M Fls-PAPL in PBS. Mixing was accomplished by inversion after each addition. The degree of polarization, P, was calculated from

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \tag{1}$$

where I_{VV} and I_{VH} are the relative intensities of the vertical and horizontal fluorescent components using vertically polarized exciting light. G is given by I_{HV}/I_{HH}, the ratio of vertical and horizontal fluorescent components with horizontally polarized light, and corrects for instrumental bias. The polarization was plotted as a function of bound ligand where the fraction of ligand bound was calculated from

$$\frac{L_0}{S_0} = \frac{1}{K_0}\frac{1}{S_0}\frac{\phi}{1-\phi} + \phi \tag{2}$$

and

$$\frac{\phi S_0}{L_0} = \frac{L_b}{L_0} \tag{3}$$

where L_0 is the total ligand concentration, L_b is the concentration of bound ligand, S_0 is the total concentration of protein sites, K_0 is the average association constant, and ϕ is the fraction of protein sites occupied. This calculation ignores the heterogeneity of the antibody preparations.

The ratio mode was used as described above to obtain corrected excitation and uncorrected emission spectra. Concentrations of fluorophores were selected to abrogate inner filtering (maximum OD < 0.05). Absorption spectra were obtained by using a Cary 15 spectrophotometer with samples at ambient temperature and an OD range of 0 to 0.1.

Fluorescence Lifetime Measurements. Fluorescence decay curves were obtained by the monophoton counting technique. The basic instrument contained a commercially available sample chamber, flash lamp, and phototube assembly (Photophysics, Ontario, Canada), time of flight electronics (Ortec, Inc., Oak Ridge, TN), and multichannel analyzer (LeCroy Model No. 3001) interfaced to a desk-top calculator (Hewlett-Packard 9825A with 9862A plotter). Four sample cuvettes and four emission filters could be selected under program control. Data recording of sample emission and reflected light from the exciting pulses was alternated, with the emission filter present and absent, respectively. This procedure provided profiles for the sample emission and the exciting flash taken over the same period of time (1 h).

The sample excitation flash was from a thyrotrongated arc through a 340-nm interference filter (35 nm full width at half-light). The emission filter was a 420-nm Schott cutoff filter. For samples which contained fluorescein, a second filter was added (Baird-Atomic 57018F) in order to eliminate signals from fluorescein emission (above 490 nm).

Data analysis was carried out on the desk-top calculator using the Laplace transform method of Gafni et al. (1975). The Dns decay curve was analyzed for the best fit to a two-exponential decay. Both the mathematical parameter S' which occurs in the analysis program and the overall time delay of the data with respect to the lamp flash were allowed to vary until the best fit of the calculated curve to data points was obtained in each case.

The rate of electronic energy transfer (k_T) due to dipole–dipole interactions is given by (Stryer, 1978)

$$k_T = R^{-6}K^2Jn^{-4}(8.71 \times 10^{23}\ \text{s}^{-1}) \tag{4}$$

with the efficiency of such transfer represented as

$$E = R^{-6}/(R^{-6} + R_0^{-6}) \tag{5}$$

R_0 is the distance in angstroms at which the probability of energy transfer equals the probability of excited-state decay by radiative or radiation-less processes and is

$$R_0 = (JK^2Qn^{-4})^{1/6}(9.7 \times 10^3) \tag{6}$$

The remaining variables are defined as follows: R, the distance in angstroms between the centers of the donor and acceptor chromophores; n, the index of refraction of the medium between donor and acceptor, taken to be 1.4; K^2, the orientation factor for dipole–dipole transfer; Q, the donor fluorescence quantum yield in the absence of energy transfer. The spectral overlap integral (J) is defined by

$$J = \frac{\int F(\lambda)\epsilon(\lambda)\lambda^4 \, d\lambda}{\int F(\lambda) \, d\lambda} \quad (7)$$

where λ is the wavelength, $F(\lambda)$ is the relative fluorescence intensity of the donor as a function of λ, and $\epsilon(\lambda)$ is the molar extinction coefficient of the acceptor as a function of λ. Numerical values for J were obtained by digitizing the steady-state fluorescence emission spectrum for antibody-bound Dns and the absorption spectrum for each acceptor moiety by using the same Hewlett-Packard 9825 calculator with 9872 plotter that was used in the analysis of the nanosecond decay curves.

Results

Anti-Dns IgG. The rabbit anti-Dns IgG used in the preparation of the covalent hybrid anti-Dns/antilactose IgG was eluted from a Dns-BSA–Sepharose 4B immunoadsorbent with 1.0 M propionic acid and 4 M urea and chromatographed on Sephadex G-200 in PBS. Both specific protein fluorescence quenching, with a maximum quench of approximately 60%, and ligand fluorescence enhancement were observed upon addition of Dns-lysine. The total number of active combining sites was obtained graphically by extrapolation of the initial, linear portion of an enhancement binding curve. The protein concentration was calculated from the optical density at 280 nm with $E_{1cm}^{1\%}$ equal to 14.4 for rabbit IgG. A comparison of these values showed that 80–85% of the total combining sites remained active. The average association constant for this heterogeneous rabbit anti-Dns IgG preparation with Dns-lysine was 19.0×10^6 M^{-1} by the fluorescence enhancement technique described by Parker et al. (1967). An estimate of the quantum yield of antibody-bound Dns-lysine was made by comparing the area of the uncorrected emission spectrum with the spectra of Dns-lysine in absolute ethanol (quantum yield = 0.33) and anhydrous dioxane (quantum yield = 0.45) as relative standards (Parker et al., 1967). This comparison was made by the relative weights of the emission spectra graphs (relative intensity vs. wavenumber). The antibody-bound ligand had a quantum yield of 0.57 ± 0.05 based on these standards. The blue shift in emission as well as the increased quantum yield which accompanies antibody binding permitted selective monitoring of the bound Dns donor.

Spectral Properties of Lactosyl Ligands. The absorption spectrum of the *p*-[*p*-(dimethylamino)benzeneazo]phenyl β-lactoside (Lac-Dye) in PBS at pH 7.2, shown in Figure 1, is characterized by a maximum at 444 to 445 nm with a shoulder around 400 nm (Karush, 1957). Ghose & Karush (1974) compared the difference spectra of free Lac-Dye and bound Lac-Dye for several homogeneous rabbit antilactose IgG's purified by preparative isoelectric focusing of antibody from rabbits with restricted antilactose responses. They found hyperchromicity as a general feature although each antibody exhibited a distinctive difference spectrum. The rabbit antilactose IgG used for the preparation of covalent hybrid molecules was a heterogeneous population which, upon binding the Lac-Dye, produced a 4- to 5-nm red shift as well as the expected hyperchromicity at the absorption maximum of the azo compound. Since the difference in the absorption spectra of bound and free Lac-Dye was less than 5% at any wavelength, the values for free Lac-Dye were used to calculate the spectral overlap integral (J) for this chromophore in relation to the emission spectrum of Dns-lysine bound to rabbit anti-Dns IgG. Assuming a molar extinction coefficient of $\epsilon_{445} = 2.49 \times 10^4$ for Lac-Dye, $K^2 = 0.667$, and a quantum yield of 0.57 for bound Dns-lysine, we calculated that $J = 7.22 \times 10^{-14}$ $cm^6/mmol$ and $R_0 = 42.9$ Å. The absorbancy of Lac-Dye in the 250–350-nm region also makes it a candidate for protein fluorescence quenching. As seen in Figure 2, Lac-Dye is an effective quencher of rabbit antilactose IgG, with a Q_{max} = 56.5% for this preparation.

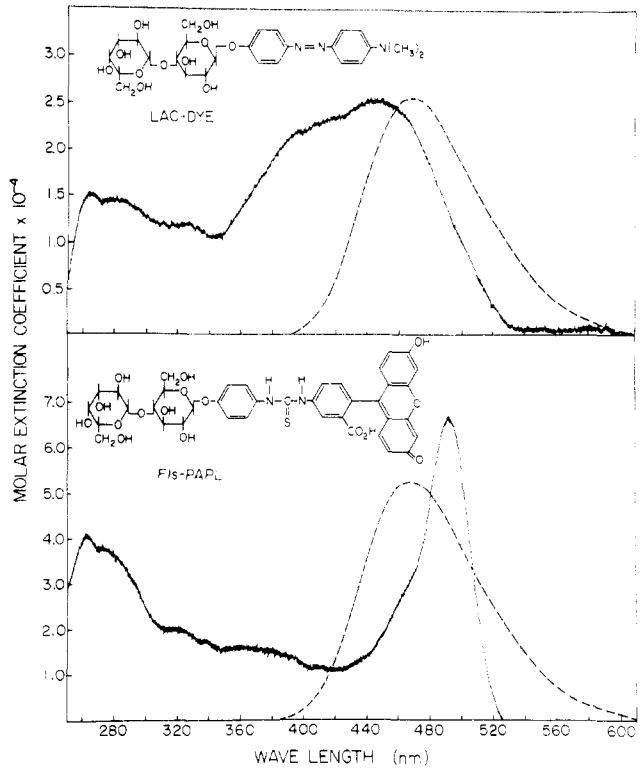

FIGURE 1: Top panel shows the structure and absorption spectrum of Lac-Dye (solid line) in PBS. $\epsilon_{445} = 2.49 \times 10^4$. Bottom panel shows the structure and absorption spectrum of Fls-PAPL (solid line) in PBS. $\epsilon_{443} = 6.76 \times 10^4$. The uncorrected emission spectrum, in relative fluorescence units, of 0.96×10^{-6} M Dns-lysine bound to 9.0×10^{-6} M rabbit anti-Dns IgG sites in PBS (excitation at 340 nm) is shown in both panels (---). More than 98% of the ligand was bound to antibody.

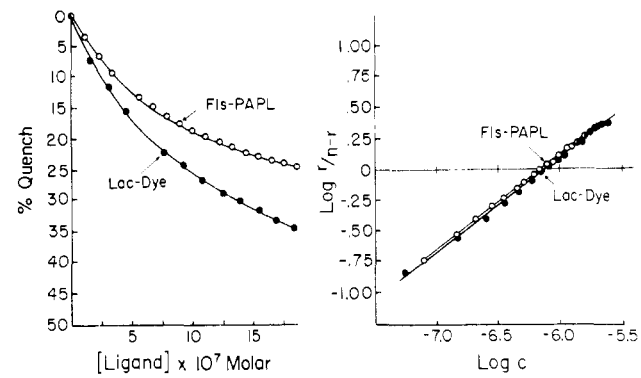

FIGURE 2: (Left) Protein fluorescence quenching by Lac-Dye and Fls-PAPL with 4.1×10^{-7} M rabbit antilactose IgG (third course) at 22 °C in PBS. Excitation wavelength was 295 nm and the emission was measured at 330 nm. Q_{max} was 40.3 and 56.5% for Fls-PAPL and Lac-Dye, respectively. (Right) Sips analysis yielding log K_0 = 6.13 and a heterogeneity index of 0.75 for Fls-PAPL and log K_0 = 6.11 and a heterogeneity index of 0.76 for Lac-Dye.

The synthesis of Fls-PAPL extends the work of Gopalakrishnan & Karush (1975) by coupling a fluorescent secondary sensor to the lactosyl moiety. The absorption spectrum of Fls-PAPL in PBS at pH 7.2, shown in Figure 1, is characteristic of hydroxyxanthenone derivatives with an absorption maximum at 492 to 493 nm. Whereas it has been shown that rabbit antifluorescein IgG produces a 5–10-nm red shift in the

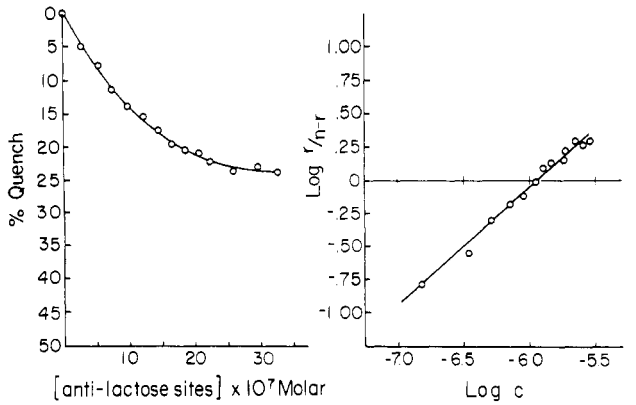

FIGURE 3: (Left) Quenching of Fls-PAPL fluorescence by titration with rabbit antilactose IgG (third course) at 22 °C in PBS. The excitation wavelength was 480 nm and emission was measured at 510 nm. Q_{max} was 35.6%. (Right) Sips analysis yielding log K_0 = 5.93 and a heterogeneity index of 0.91.

absorption spectrum of bound fluorescein (Watt & Voss, 1977), the binding of antilactose antibody to Fls-PAPL caused a small but discernible 2- to 3-nm red shift in the absorption spectrum of the bound species. Employing the spectrum of the free Fls-PAPL for the calculation of the overlap integral with the antibody-bound Dns-lysine, we obtained $J = 14.68 \times 10^{-14}$ cm^6/mmol and $R_0 = 48.3$ Å using $\epsilon_{493} = 6.76 \times 10^4$ for Fls-PAPL and $K^2 = 0.667$. Since fluorescein has been shown to be an effective quencher of protein fluorescence (Watt & Voss, 1977), Fls-PAPL was titrated with rabbit antilactose IgG and found to quench protein fluorescence with a Q_{max} = 40.3% for this preparation. The similarities of the calculated binding parameters despite quite different Q_{max} values for Lac-Dye and Fls-PAPL suggest that the phenyl β-lactoside portion of the molecule makes the major contribution to the binding energy.

These results establish, therefore, the utility of the chromophores described above as acceptors of electronic energy from both specific protein tryptophanyl residues as well as synthetic fluorophores such as Dns. In addition, they define the position of the acceptor as immediately adjacent to the antigen-specific combining site of IgG.

Mobility of the Energy Acceptor. The similarities between the binding parameters for rabbit antilactose IgG with the above two lactosyl derivatives [as well as Dns-PAPL, pyrene–PAPL, and fluoranthenyl-PAPL (unpublished data)] indicate that there was minimal interaction between the secondary sensor and the surface of the IgG molecule. We utilized the fluorescence properties of Fls-PAPL to investigate this point further. It has been reported that fluorescein emission is extensively quenched (~90%) when bound by antifluorescein antibody (Werner & Cathou, 1971; Watt & Voss, 1977). Two mechanisms for this quenching have been proposed. The first was based on the view that the local environment of the protein, with an overall lower dielectric constant than the aqueous compartment, would tend to protonate one of the hydroxyxanthenone oxygens, thus destroying the symmetry of its resonant fluorescent form (Dandliker, 1977). The second (Watt & Voss, 1977) noted that the observed quench is a complex combination of both collisional quenching and the environmentally stabilized, nonfluorescent form of the probe in which tryptophanyl and tyrosyl residues would play an important role. We observed a diminution of the 510-nm emission of Fls-PAPL but not to the extent reported with the fluorescein–antifluorescein system. The maximum observed quench was approximately 24% (Figure 3), although double-reciprocal plots of percent quench vs.

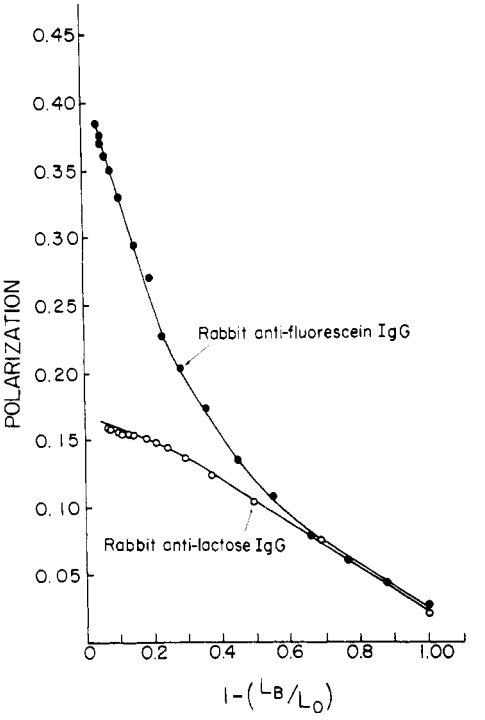

FIGURE 4: Steady-state fluorescence polarization of Fls-PAPL at 20 °C in PBS at an initial concentration of 0.92×10^{-6} M as a function of the fraction bound by either rabbit antilactose or antifluorescein IgG. L_0 is the total concentration of ligand; L_B is the the concentration of bound ligand. Double-reciprocal plots of these data extrapolate to polarization values of 0.44 and 0.17 for antifluorescein and antilactose, respectively. The excitation wavelength was 480 nm and emission was measured at 510 nm. The association constant of Fls-PAPL for antilactose IgG was 1.30×10^6 M^{-1} and for antifluorescein was 18.6×10^6 M^{-1}.

antibody site concentration indicated the average Q_{max} would be closer to 35%. To delineate the extent of rotational freedom of the chromophore when bound by rabbit antilactose IgG, we performed a steady-state fluorescence polarization experiment in which the polarization of Fls-PAPL was monitored in PBS at 20 °C as a function of increasing concentrations of either rabbit antilactose or antifluorescein IgG. Figure 4 shows that the extrapolated values for polarization of the bound hapten are 0.44 and 0.17 for antifluorescein and antilactose, respectively. Since it appears that the fluorescein group is immobilized in the combining site of the antifluorescein antibody, we infer that the fluorescein moiety of the Fls-PAPL has rotational mobility independent of the segmental flexibility inherent in the IgG molecule.

Preparation and Characterization of Covalently Linked Hybrid IgG. In the preparation of our hybrid IgG, we sought to reassociate the rabbit IgG into a configuration similar to the native molecule by re-forming all of the interchain disulfides. We followed the method of Hong & Nisonoff (1965), excluding the alkylating agent. This modification permitted the reoxidation of reduced disulfides. In the procedure of Hong & Nisonoff (1965), the low pH resulted in half-molecules (HL) with insignificant separation of the homologous heavy–light chain combination. Accordingly, the mixture of rabbit antilactose and anti-Dns IgG was reduced with 2-mercaptoethanol, acidified to rearrange half-molecules, and dialyzed to increase the pH and promote reoxidation. The molar ratio of rabbit antilactose to anti-Dns IgG was 70:30, resulting in the following theoretical bivalent combinations: 9% anti-Dns/anti-Dns, 49% antilactose/antilactose, and 42% anti-Dns/antilactose. After extensive dialysis, the hybrid prepa-

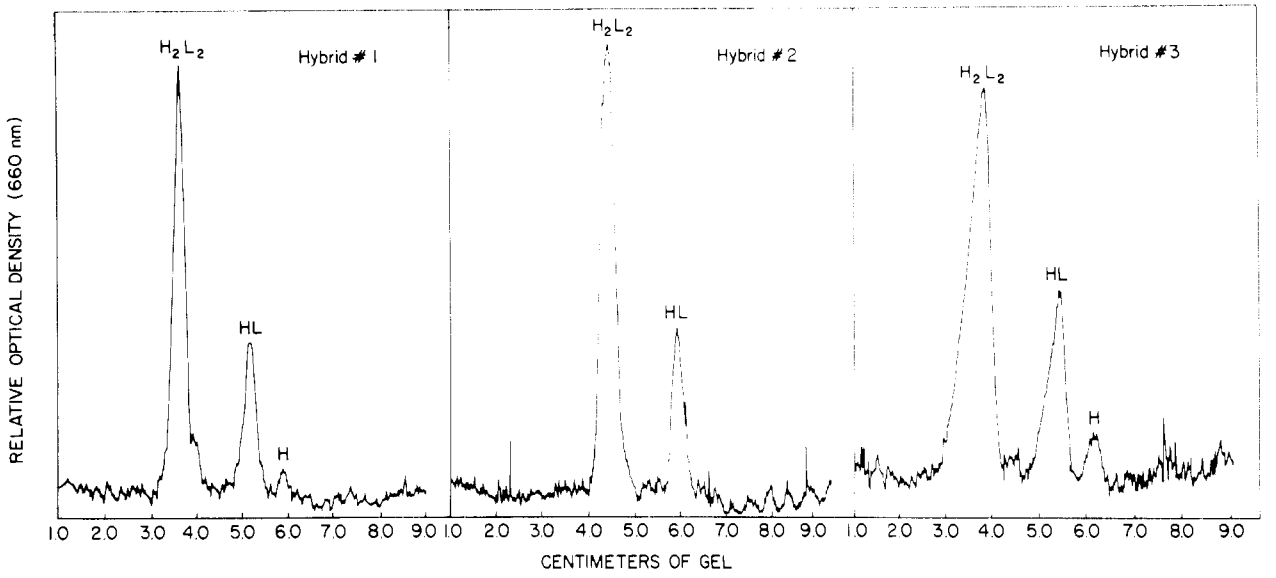

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis linear scans of rabbit covalent anti-Dns/antilactose IgG preparations stained with Coomassie Brilliant Blue R-250. The positions of intact IgG (H_2L_2), heavy (H), and light (L) chains were established for each gel by comparison to protein standards and bromophenol blue tracking dye positions. The average of triplicates for the mass of the H_2L_2 species is 79.9% for hybrid 1 and 79.1% for hybrid 2. These preparations were used for fluorescence measurements. Hybrid 3, containing rabbit [I^{125}]anti-Dns IgG, was not used for spectroscopic measurements (see Figure 6). The analysis of the hybrids was performed after adsorption to Lac–Sepharose and elution with 0.2 M lactose.

rations were chromatographed on a Lac–Sepharose column. This step resulted in the removal of the anti-Dns/anti-Dns species from the hybrid preparation. This was followed by the nondenaturing elution of antibody with lactose, yielding IgG molecules in which at least one active antilactose combining site must be present. Since the lactose-eluted hybrid preparation demonstrated both fluorescence enhancement of Dns-lysine and protein fluorescence quenching with Fls-PAPL, the anti-Dns combining site must be associated with an antilactose site. The theoretical distribution of hybrid pairs in the lactose-eluted hybrid preparations would be 54% antilactose/antilactose and 46% anti-Dns/antilactose, with the anti-Dns comprising 23% of the total combining sites.

The extent of reoxidation to a covalent IgG form (H_2L_2) was quantitated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 5). Linear scans of stained tube gels indicated that 79 to 80% of the total protein was in the covalent (H_2L_2) form, with the second component comprised of half-molecules (HL). The half-molecule (HL) component would exist as a bivalent noncovalent IgG (HL–HL) in physiological buffer systems.

Since the anti-Dns/antilactose hybrid constituted slightly less than half of the total protein, the presence of the noncovalently associated hybrid IgG raised the possibility that the anti-Dns/antilactose species was disproportionately represented in this form. That is, a restriction on the re-formation of the disulfide of hybrid IgG from two outbred rabbits might result in the noncovalent form (HL–HL) being comprised primarily of the anti-Dns/antilactose hybrid. To test this possibility we trace radioiodinated rabbit anti-Dns IgG with ^{125}I prior to hybrid formation. Since the maximum value for ^{125}I atoms per IgG molecule was 0.22, denaturation of the protein due to excessive iodination of tyrosines was unlikely. When the radiolabeled hybrid preparation, prepared as previously described, was chromatographed on Lac–Sepharose, the lactose-eluted portion comprised 91.5% of the total protein (by OD_{280}) and 72% of the radioactivity. These values in excellent agreement with the theoretical values of 91.0 and 70%, respectively. Comparison of linear gel scans for protein (Figure 5) and the γ emission (Figure 6) demonstrated that the distribution of the label was essentially identical with the distribution of protein. The conclusions, therefore, are as follows: (1) there is no restriction to the formation of inter-heavy-chain disulfide bonds between these preparations of rabbit anti-Dns and antilactose IgG and (2) 79 to 80% of the anti-Dns antilactose hybrids used in the energy-transfer analysis were in a bivalent, covalently linked form.

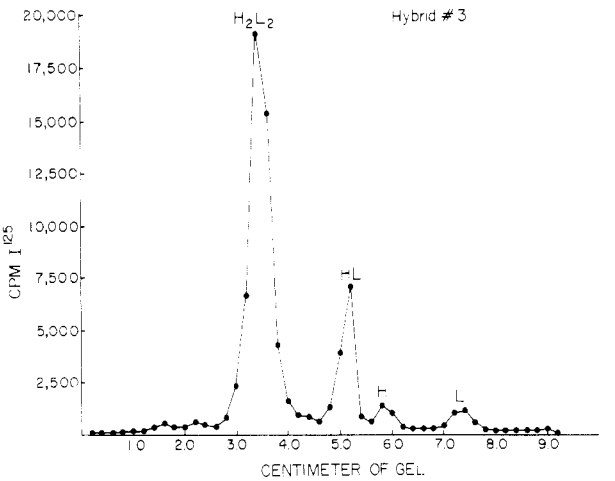

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of hybrid 3 for which [I^{125}]anti-Dns rabbit IgG was used for the preparation of the anti-Dns/antilactose covalent hybrid. The results demonstrate a similar distribution of radioactivity and protein (see Figure 5). For hybrid 3, 71.5% of the anti-Dns was in the covalent H_2L_2 form. The analysis was performed after adsorption to Lac–Sepharose and elution with 0.2 M lactose.

Fluorescence Analysis. Steady-state fluorescence analysis of energy transfer was evaluated using hybrid preparations 1 and 2, with rabbit anti-Dns IgG as a control. The concentrations of anti-Dns combining sites for each preparation obtained by extrapolation of fluorescence enhancement curves was set at 8.5×10^{-7} M. The linearity of the fluorescence enhancement binding curves at these protein concentrations verified that the concentration of anti-Dns binding sites was at least 10-fold greater than the reciprocal of the association constant ($K_0 > 10^7$ M^{-1}). Dns-lysine was added to each protein

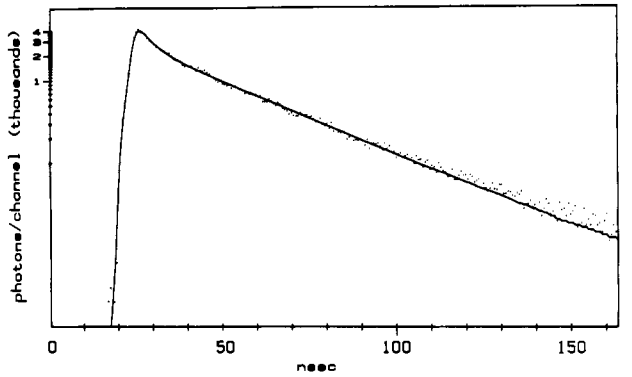

FIGURE 7: Fluorescence decay curve for Dns-lysine bound to hybrid preparation 1 in the presence of Lac-Dye. The number of photons collected per channel (0.64 ns/channel) is plotted on a logarithmic scale as a function of time. Values for each of the 256 channels are shown as discrete points. The continuous curve is the decay calculated by using the experimental lamp flash and fluorescent lifetimes of 24.0 and 3.4 ns. The fraction of the total emission contributed by these two components (given by the product of the amplitude and lifetime of each) is 84 and 16%, respectively. These parameters were obtained by the Fourier transform method of Gafni et al. (1975) by analyzing for the best two-component fit. The lamp curve was shifted 0.30 ns relative to the decay curve. The root mean square difference between the calculated value and the experimental value at each channel was 40 photons or 0.99% of the peak number of photons per channel.

solution such that approximately half of the anti-Dns sites were occupied. The percent of ligand bound is estimated to be at least 90%. Samples were excited at 340 nm and monitored at 470 nm for Lac-Dye titrations or 460 nm for Fls-PAPL (due to overlapping emission of Fls-PAPL). Because of the blue spectral shift and dramatic increase in quantum yield of antibody-bound Dns-lysine, the unbound species contributed less than 1% to the emission signal. (These calculations are further supported by the linearity of the long-lifetime component shown in Figure 7.) Lac-Dye or Fls-PAPL was titrated into each protein solution (in duplicate) such that at the conclusion at least 95% of the antilactose combining sites were filled with acceptor. An anti-Dns solution was used as a control to correct for dilution, for nonspecific attenuation of emission, and, in the case of Fls-PAPL, for overlapping emission of the acceptor molecule. The average ratio (four titrations) of the emission signals from the hybrids to the control preparations was 0.98 ± 0.01 with Lac-Dye and 0.97 ± 0.01 with Fls-PAPL. With the ±3% error inherent in steady-state measurements, these results demonstrate the absence of energy transfer in the hybrid molecules.

Fluorescence lifetime decay curves were obtained by the monophoton counting method for Dns-lysine bound to rabbit anti-Dns IgG or to rabbit covalent hybrid preparations. Measurements were made either in the presence or in the absence of the lactosyl chromophores, and the data were analyzed for two exponential decays. Both a short-lifetime (τ_s) component of 2–4 ns and a longer component (τ_l) with a decay time of approximately 24 ns were observed. The values obtained for τ_l depended slightly upon the emission wavelengths being monitored. For photons emitted at wavelengths greater than 420 nm, an average value of 23.8 ns was obtained for τ_l, whereas the positioning of a 470-nm filter in the emission path resulted in a shift (less than 2%) of τ_l to 24.1 ns. These values of τ_l are within the range of lifetimes previously reported for Dns-lysine bound to rabbit anti-Dns IgG (23.1–23.9 ns) and analyzed for a single exponential decay (Werner et al., 1972; Yguerabide et al., 1970).

The analysis of our data for bound Dns-lysine gave the results shown in Table I. Virtually identical values for τ_l were

Table I: Fluorescence Lifetime Measurements on Bound Dns-lysine

sample	acceptor	optical filter[a]	long-lifetime component[b] (ns)
anti-Dns, hybrid 1, hybrid 2	none	C	23.8 ± 0.3 (3)
anti-Dns, hybrid 2	none	C + F	24.1 ± 0.2 (2)
anti-Dns	free Lac-Dye	C	24.0 (1)
anti-Dns	free Fls-PAPL	C + F	24.7 (1)
hybrid 1, hybrid 2	bound Lac-Dye	C	23.6 ± 0.3 (3)
hybrid 2	bound Fls-PAPL	C + F	24.4 (1)

[a] Filter C = 350-nm interference excitation filter plus 420-nm cutoff emission filter. Filter F = 490-nm interference filter added to the emission path to eliminate Fls fluorescence. [b] Mean ± standard deviation, in nanoseconds, with the number of measurements in parentheses.

obtained for the three following situations: (1) Dns-lysine bound to native or covalent hybrid IgG antibody with no acceptor present; (2) Dns-lysine bound to native rabbit anti-Dns IgG in the presence of unbound acceptor; (3) Dns-lysine bound to covalent hybrid antilactose/anti-Dns in the presence of antibody-bound acceptor. The standard deviations of repetitive experiments were less than 2% of the means, and the values covered a total range of approximately 0.6 ns. This deviation is within the estimates of the sensitivity of the technique. Since the values for τ_l in the presence or absence of bound acceptor are within one standard deviation, it is clear that no detectable quenching has occurred due to energy transfer between these donor–acceptor pairs positioned in adjacent antigen combining sites. Comparable results were obtained by using either Lac-Dye or Fls-PAPL as the acceptor although, as previously mentioned, the observed lifetimes appear to be approximately 3% greater when using Fls-PAPL.

Discussion

Our interest in the dynamics of the conformation of IgG stems from previous studies on the significance of antibody multivalency and the structural features of the molecule which favor multivalent interactions. The architecture of the IgG molecule provides a distinctive structure particularly effective for bivalent interactions. The requirements for the functional utilization of multivalence are (1) intramolecular symmetry which results in multiple, identical antigen binding sites and (2) segmental flexibility of the Fab portions of the molecule. The conjunctive effect of these properties is the capacity of IgG to adjust the separation and orientation of its binding sites to identical reiterated antigenic determinants. The biological utility of the resulting multivalent interaction is undoubtedly linked to the enhanced affinity associated with multivalent binding (Karush, 1978). The experiments analyzed in this communication seek to define the spatial constraints which the immunoglobulin structure imposes upon the relative motion of the Fab arms of IgG.

The use of Lac-Dye and Fls-PAPL in these studies extends previous experience with *N*-(*N*$^{\epsilon}$-Dnp-L-lysyl)-*p*-aminophenyl β-lactoside (EK compound). The EK compound and a bis-(EK) ligand have been used to contrast the interaction of antilactose antibody with monovalent and divalent ligands (Karush et al., 1979; Gopalakrishnan & Karush, 1975). The studies reported in this communication show that the *N*-[(dimethylamino)phenylazo]phenyl and fluorescyl chromopores can also serve as quenchers of protein fluorescence. In addition, there was also a perturbation of the spectral properties of the sensor itself. Our experience with several

fluorescent derivatives of *p*-aminophenyl β-lactoside (PAPL) has shown that changes in the excitation and emission spectra are not observed when the average association constant is of the order of 10^5 M^{-1} or less. For 10^6 M^{-1} or greater, however, the emission properties of the sensor are altered (unpublished results). The changes are consistent with the previously observed and more drastic spectral changes seen when the antibody was directed toward the probe itself.

The utilization of a covalent hybrid IgG for this study was critical because the single rabbit IgG inter-heavy-chain disulfide bond is the dominant factor in maintaining the integrity of the $C_\gamma2$ module. The reason for incomplete reoxidation of rabbit IgG during the preparation of the covalent hybrid is unclear, but two nonexclusive possible explanations can be offered. First, in studies by Litske & Dorrington (1974) on the reoxidation of human IgG_1 and IgG_4, it was observed that after reduction IgG_1 reoxidized back to the covalent (H_2L_2) form, while with IgG_4 substantial levels of the half-molecule (HL) persisted even after extended incubation. In addition, the work of Florent et al. (1973) suggests the presence of subclasses of rabbit IgG, which differ in the $C_\gamma2$ region of the molecule. There exists, therefore, the possibility that some subgroups inefficiently reoxidize inter-heavy-chain disulfide bonds and/or certain hybrid combinations of rabbit IgG subgroups would be incompatible for inter-heavy-chain reoxidation. The second possibility arises as the result of the generation of other oxidation products (i.e., R–SOH, R–SO_3H). The second hypothesis is favored since no free sulfhydryls could be detected by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).

Some of the investigations which have provided recognition of the critical structural role of the inter-heavy-chain disulfide have already been noted. In addition, Romans et al. (1977) have proposed that upon reduction and alkylation of human IgG the $C_\gamma2$ module spontaneously dissociates, each $C_\gamma2$ domain gains independent modes of movement, and the molecule acquires additional segmental flexibility. Their experiments involved the conversion by mild reduction of "incomplete" or nonagglutinating human anti-D, anti-c, and anti-E alloantibodies (IgG) to direct agglutinins. This result was attributed to the conversion of the IgG molecule to a configuration which permitted cell bridging due to an increase in the distance which the chemically modified immunoglobulin could span and was interpreted as evidence for the restriction of segmental flexibility by the inter-heavy-chain disulfide bonds. They estimated that the increased separation of the combining sites could be as much as 80–140 Å.

More recently, another aspect of the proximity of the Fab arms in solution has been examined by Schumaker et al. (1979). The distribution of the sizes of the complexes formed between IgG antibody and a small bivalent ligand was established from the analysis of sedimentation patterns. The inference was drawn that the Fab arms are not subject to significant hindrance when their angle of separation is greater than 60°. On the other hand, the formation of antibody dimers cross-linked by bivalent ligand is impeded by a substantial energy barrier because of the reduced angle required for its formation. These experiments provide an independent demonstration of steric restrictions against the close approach of the combining sites of the IgG molecule.

The results of our measurements are in accord with the view that the antibody molecules in solution are in a very open Y- or, possibly, T-shaped configuration (Cathou, 1978). The analysis of an energy-transfer experiment depends, however, upon the relative orientations of the donor emission and acceptor absorption dipoles, as well as the distance between them. Therefore, a reliable estimate of the distance separating the two chromophores cannot be obtained until the issue of the orientational factor has been resolved. Mathematically, this factor is K^2 in eq 4 and 6 and is relevant to the calculation of the "scale factor" R_0. For single, well-defined, donor–acceptor pairs, K^2 can vary from 0 to 4 and is equal to 2/3 for a completely random distribution of orientations.

Haas et al. (1978) have calculated the probable error for various donor–acceptor pairs if the value $K^2 = 2/3$ is used in the calculation of R_0 instead of explicitly averaging over the angles of orientation in the exact mathematical expression. In particular, they emphasized that the presence of two or more dipole moments in the donor and/or acceptor or motion during the excited-state lifetime (which are formally identical) will create an averaging effect and prevent very small values of K^2 from being possible. Both Haas et al. (1978) and Dale & Eisinger (1975) have pointed out that an analysis which assumes $K^2 = 2/3$ will not be in serious error as long as the correct K^2 value is not extremely small. The former authors have calculated the maximum possible and maximum probable errors for systems having given polarizations of the emitting and absorbing chromophores.

By this analysis the orientational dependence in the efficiency of energy transfer for the anti-Dns/antilactose covalent hybrid system is minimized for several reasons. First, in a study of the linear and circular fluorescence polarization of a series of Dns ligands bound to rabbit anti-Dns antibody, a pronounced variation of the linear polarization across the emission band was observed, indicating a variety of transitional dipole moments with different spatial directions (Schlessinger et al., 1974). This multiplicity of emission dipoles is further randomized by being bound to an IgG which exhibits segmental flexibility. The observed linear polarization of the fluorescence under the conditions of our experiments was 0.3 in the study by Schlessinger et al. (1974). Second, steady-state polarization analysis of Fls-PAPL bound to antilactose IgG indicates that the fluorophore is not rigidly held on the surface of the protein but rather exhibits independent rotational mobility. This mobility, combined with the degeneracy of the absorption dipole due to the symmetry of the chromophore, serves to randomize the absorption dipole of the acceptor at a position adjacent to the combining site (Athey & Cathou, 1977). Third, in a similar system energy transfer was observed between an anti-Dns-bound Dns donor and a fluorescein acceptor covalently linked to a reduced interchain disulfide. The distance calculated was in good agreement with X-ray crystallographic data (Bunting & Cathou, 1973). Finally, we have utilized the theoretical analysis of the probability of energy transfer with probes involving electronic transitions of mixed polarity (Haas et al., 1978). On the basis of the observed steady-state polarizations of the bound Dns-lysine and the bound Fls-PAPL, the probable error in the evaluation of the separation for this donor–acceptor pair would not exceed 11%.

An estimation of the minimum distance between the binding sites of IgG encounters difficulty, however, because of the segmental flexibility of the Fab arms. This flexibility results in a distribution of distances as well as a time-dependent variation for each molecule during the lifetime of the excited state of the energy donor. In order to estimate the significance of these dynamic factors, we consider three models which involve different simplifying assumptions with respect to these factors.

Model 1. If we ignore flexibility completely and assume that all the donor–acceptor pairs are characterized by the same

distance R and same angle θ between the Fab arms, the observation that $|\tau - \tau_0| \leq 0.03\tau_0$ means that $R \geq 1.8R_0$ ($R \geq 86$ Å) and $\theta > 66°$. This model clearly overestimates the angle of minimum separation due to the assumption that all molecules can be characterized by the same angle. It would not be possible simply to consider this to be an average angle of separation since the energy-transfer process depends on the sixth power of the separation.

Model 2. The simplest model which removes the restriction that there is a single angle between Fab arms in all molecules and begins to take account of the IgG flexibility is a static model in which orientations are equally probable for all angles $\theta > \theta_{min}$. For simplicity in the calculation we have ignored the orientation of the two Fab arms with respect to the Fc region and calculated the mean values for the lifetime $\langle\tau\rangle$ and the fluorescence decay rate $\langle 1/\tau\rangle$. In the case of $\langle\tau\rangle$ we require

$$\frac{\langle\tau\rangle}{\tau_0} = (1 + \cos\theta_{min})^{-1}\int_{\theta_{min}}^{\pi}(\sin\theta)\left(\frac{R^6}{R_0^6 + R^6}\right)d\theta \geq 0.97 \quad (8)$$

If $R_0 = 48$ Å and the length of the Fab equals approximately 80 Å, the values of θ_{min} consistent with this requirement are $\theta_{min} > 37°$.

This value for θ_{min} probably underestimates the angle of closest approach for two reasons: (1) steric hindrance due to the Fc region has been ignored and this would reduce the number of allowed orientations in space; (2) the motion of the Fab arms which would carry some pairs closer together during a fluorescence lifetime and increase the quenching corresponding to a given value of θ_{min} has also been ignored.

Model 3. We can include diffusion in an approximate manner by considering donor molecules which are closest to acceptors ($\theta \sim \theta_{min}$) as a separate "species" having a lifetime τ_Q and assuming that the unquenched remainder is converted into the quenched form with a rate constant given by the diffusion time, $k_D^{-1} = 33$ ns (Yguerabide et al., 1970). The initial concentration of quenched donor is taken to be zero, and, for simplicity, the back-diffusion away from θ_{min} and toward the unquenched form is omitted. The solution of the coupled differential equations for the two forms yields the following expression for the observed fluorescence decay

$$I(t) \propto \left(k_0 - \frac{k_D k_Q}{k_0 + k_D - k_Q}\right)e^{-(k_0+k_Q)t} + \frac{k_D k_Q}{k_0 + k_D - k_Q}e^{-k_Q t} \quad (9)$$

where k_0 is the unquenched rate ($=\tau_0^{-1}$) and k_Q is the quenched rate ($=\tau_Q^{-1}$).

In order to ascertain what τ_Q in a decay law of the above form would actually be detected by our data analysis curve fitting program, we mathematically simulated decays using eq 9. A measured decay curve was modified by subtracting out the long-lifetime component and replacing it with the two-component decay given by eq 9, simulated by using the experimental lamp flash curve. The new decay curve retained the noise and short-lifetime component from the original. When the data analysis was carried out, it was found that for $\tau_Q < 22.5$ ns the analysis yielded lifetimes which were changed from the original by more than the uncertainty in our measurements: τ_{calcd} was < 23.2 ns in comparison with 24.0 ns for τ_0. If this value of τ_Q corresponds to the minimum distance between binding sites, we infer that $R_{min} = 75.4$ Å and $\theta_{min} = 50°$.

This estimate of the closest approach is subject to two principal sources of error. The first is the omission of diffusion away from θ_{min}. This factor becomes important at longer times and, if included, would tend to decrease the estimate for θ_{min}. The second approximation is the omission of quenching in the starting distribution of positions as was treated in model 2. Inclusion of this factor would tend to increase the value for θ_{min}.

On the basis of the model calculations, we conclude that the twin observations of no energy transfer and flexibility about the IgG hinge region together imply that there is an energy barrier against the mobile Fab arms approaching each other in solution closer than some distance R_{min}. From the above estimates of the sensitivity of our technique to a possible distribution of fluorescence lifetimes due to energy-transfer quenching, we estimate that R_{min} must be at least 55–70 Å, corresponding to $\theta_{min} = 40$–$50°$.

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Immunochemical Specificity of the Combining Site of *Wistaria floribunda* Hemagglutinin†

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ABSTRACT: The specificity of *Wistaria floribunda* hemagglutinin, purified by adsorption on insoluble polyleucyl hog gastric mucin blood group A + H (PL-hog A + H) substance, elution with lactose, and gel filtration on Sephadex G-200, was studied immunochemically by quantitative precipitin and precipitin inhibition assays. The purified hemagglutinin, with a molecular weight of 68 000 and an isoelectric point of 5.0, was homogeneous electrophoretically and immunochemically and was made up of two covalently linked subunits of molecular weight 32 000. It was precipitated by blood group A, BI, Le^a, and precursor I substances and by B substance subjected to mild acid hydrolysis (P1) but poorly by blood group B and H substances. It was also precipitated by asialoorosomucoid (ASOR) but not by agalactoorosomucoid (AGOR) and inactive antifreeze glycoprotein. Inhibition assays with various monosaccharides, glycosides, and oligosaccharides indicate that the hemagglutinin is most specific for terminal nonreducing α-linked DGalNAc. The best inhibitor was the disaccharide DGalNAcα(1→6)DGal which was 8.8 times more potent than DGalNAc. The A-active di-, tri-, and pentasaccharides were 3.3, 3.8, and 12.0 times less potent than DGalNAcα(1→6)DGal, respectively. Among the glycosides of DGalNAc tested, pNphβDGalNAc was most potent. It was 6.0 times more active than DGalNAc but 1.5 times less active than DGalNAcα(1→6)DGal. Although the best inhibitor was the α-linked disaccharide DGalNAcα(1→6)DGal and MeαDGalNAc was better than MeβDGalNAc, pNphβDGalNAc was more potent than pNphαDGalNAc. Molecular models showed pNphβDGalNAc and DGalNAcα-(1→6)DGal to be similar in shape and to differ from pNphαDGalNAc. These findings indicate that the combining site of the hemagglutinin is at least as large as a disaccharide and that hydrophobic interaction and shape are important for binding.

The hemagglutinating substances (lectins) obtained from plants and animals have specific receptor sites for glycoproteins (Kabat, 1976; Lis & Sharon, 1977; Goldstein & Hayes, 1978; Pereira & Kabat, 1979). They have been used extensively as reagents for detecting the distribution and mobility of glycoproteins on normal and malignant cell surfaces (Nicholson, 1974; Sharon & Lis, 1975) and for isolation of glycoproteins present on the cell surface (Kimura & Wigzell, 1978; Kimura et al., 1979).

Wistaria floribunda hemagglutinin and mitogen have been isolated separately from crude seed extracts by column chromatography (Toyoshima et al., 1971; Kurokawa et al., 1976) and by using immunoadsorbents (Cheung et al., 1979). The hemagglutinin is a glycoprotein which agglutinates human A, B, and O erythrocytes nonspecifically. Although some properties of the isolated hemagglutinin reported by different investigators have varied slightly, hemagglutination inhibition assays were in accord in that hemagglutination is inhibited best by DGalNAc[1] and synthetic DGalNAcα1→Tos-L-Ser

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[1] Abbreviations used: DGalNAc, 2-acetamido-2-deoxy-D-galactose; DGlcNAc, 2-acetamido-2-deoxy-D-glucose; DGal, D-galactose; DGlc, D-glucose; DFru, D-fructose; *p*, pyranoside; *f*, furanoside; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PL-hog A + H, polyleucyl hog gastric mucin A + H blood group substance; Ara, arabinose; ASOR, asialoorosomucoid; AGOR, agalactoorosomucoid; pNph, *p*-nitrophenyl; PhOH, phenol-insoluble fraction.

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