Site-Directed Labeling of a Monoclonal Antibody: Targeting to a Disulfide Bond†

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ABSTRACT: We have designed and synthesized crabescein, the first member of a class of fluorescent labels that add across disulfide bonds. Crabescein is a fluorescein derivative that reports the rotational correlation time of the immunoglobulin G (IgG) segment to which it is covalently bound. Chemical analysis of the IgG labeled with crabescein indicates that the fluorophore is inserted into the third disulfide bond (cysteine-229 of mouse IgG_{2a}) in the hinge region. The rotational correlation time of this labeled macromolecule was measured as a single exponential with a decay constant of 26.8 ns. This is in contrast to the double exponential with decay constants of 14.3 and 0.2 ns for the same IgG when labeled with fluorescein via a conventional labeling reagent in which the probe is bound to the macromolecule by one-point attachments. Thus, crabescein is the prototype of a class of fluorescent and phosphorescent probes that, by virtue of their two-point attachments to proteins, faithfully report on the dynamics of the segment of macromolecule to which they are covalently bound.

Fluorescence spectroscopy of macromolecules is used in many areas of cell biology; application of the technique can produce a unique class of information due to the combination of the inherently high sensitivity of fluorescence detection (Cundall & Dale, 1983; Lakowicz, 1983) and abundance of potential labeling targets in cells (Taylor et al., 1986; Thaer & Sernetz, 1973). Types of questions that can be addressed include the identification of antigens on cell surfaces, the study of subcellular compartmentalization (Packard et al., 1984), and the dynamics of macromolecular motion (Badea & Brand, 1979). While many of these applications require only that a molecule be labeled with a fluorescent probe at a sufficiently high level to visualize it, other applications, for example, the measurement of rotational diffusion of membrane proteins, depend critically upon the uniformity of protein labeling and the extent to which the dynamic properties of a fluorescent group accurately reflect the dynamics of the target molecules.

Typically, proteins such as antibodies are covalently labeled on the amino chain termini and the ϵ-amino groups of lysines with fluorophores containing reactive groups such as isothiocyanates, triazinates, and sulfonyl chlorides. Additionally, the sulfhydryls of cysteines as well as the carboxyls of aspartic and glutamic acids are potential targets for labeling with fluorophores. Several drawbacks are inherent in all commonly used covalent labeling procedures. First, proteins contain a multitude of comparably reactive groups; thus, labeling of proteins usually results in a population of molecules that is heterogeneous with respect to location of the fluorophore and cannot readily be resolved into chemically distinct species.

A second drawback is the autonomous rotational motion that a fluorophore may retain relative to the protein molecule. This is a consequence of the fact that the chemical linkage between commonly used fluorophores and proteins is a single covalent bond. For many classes of measurements, this is not a drawback; however, if, for example, one uses fluorescence depolarization to determine the rotational motion of a protein, the time-dependent depolarization may be dominated by the motion of the probe. Thus, the value obtained is probably not a true indicator of the rotational correlation time of the protein.

We report here the synthesis of a new labeling reagent, crabescein, that links fluorescein to proteins in a highly selective manner—across a disulfide bridge. A monoclonal antibody labeled with this reagent stains cells brightly, as does the same antibody conventionally labeled with fluorescein. However, the latter bears fluorescein at several different sites, and the rotation of the fluorophore is faster than that expected for the protein in solution. In contrast, molecules labeled with crabescein bear the fluorescein label at a single site—across a disulfide in the hinge region—and the rotational correlation time of the probe reflects the motion of the Fc region of the immunoglobulin G (IgG).

Materials and Methods

Materials

5′-Carboxyfluorescein was purchased from Calbiochem, cystamine and dithiothreitol (DTT) were from Sigma Chemical Co., diisopropylcarbodiimide and 1-hydroxybenzotriazole hydrate were from Aldrich Chemical Co., TPCK-trypsin was from Worthington Biochemical Co., high-pressure liquid chromatography (HPLC) grade solvents were from Baker and Aldrich, Bio-Gel P-10 was from Bio-Rad, and 5-(4,6-dichloro-*s*-triazin-2-yl)aminofluorescein (5-DTAF) was from Molecular Probes.

A murine cell line producing a monoclonal IgG_{2a}, denoted KE-2, was the generous gift of Dr. Roger Kennett (University of Pennsylvania). KE-2 cells were grown in Eagle's minimal essential medium, with 10% fetal calf serum. The IgG was purified from spent medium by adsorption to Affi-Gel protein A (Bio-Rad) and eluted according to the MAPS (Bio-Rad) procedure.

Methods

Synthesis. 5′-Carboxyfluorescein (3.7×10^{-5} mol) (Figure 1A), cystamine, diisopropylcarbodiimide, and 1-hydroxy-

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benzotriazole were dissolved in dimethyl sulfoxide (571 μL) in a molar ratio of 1:10:40:40, respectively, and the pH was adjusted to 10. After being mixed at room temperature for 10 min, the solution was diluted with a 0.1% trifluoroacetic acid (TFA) containing mixture of water and acetonitrile in a volume ratio of 9:1, respectively. The precipitate (mostly urea) was removed by centrifugation. The intermediate product, named procrabescein (Figure 1B), was isolated by reverse-phase HPLC (Waters Associates) on a μBondapak CN column (7.8 mm × 30 cm) (Waters Associates). The elution gradient ran from an initial composition of 10% (v/v) acetonitrile in water to a final composition of 90% (v/v) acetonitrile in water with 0.1% TFA (v/v) present throughout (Figure 1).

After removal of the HPLC solvents by lyophilization, the ninhydrin-positive compound was dissolved in an aqueous solution containing 50% (v/v) ethanol/water with dithiothreitol (DTT) in 10-fold molar excess over the procrabescein. The reduced product was separated by the same reverse-phase chromatography conditions used above (Figure 1C).

Proton nuclear magnetic resonance (NMR) (Varian XL-400), ultraviolet–visible (UV–vis) resonance (Varian Cary 219), and mass spectra (Hewlett-Packard Model 5980A) of the final product, crabescein, were then taken.

Incorporation. A mouse monoclonal antibody, KE-2, to the human major histocompatibility antigens HLA-A, -B, and -C was dissolved in phosphate-buffered saline (PBS) (pH 7.4) at a concentration of ca. 1 mg/mL. To this solution were added a 10-fold molar excess of DTT and a 5-fold molar excess of ethylenediaminetetraacetic acid (EDTA). The pH was adjusted to 10, and the solution was incubated at 37 °C for 30 min. A 100-fold molar excess of crabescein was then added, and the incubation was continued at 37 °C for an additional 10 min. Oxygen gas was bubbled through the solution, and the pH was dropped to 5.5; the solution was kept at 37 °C for ca. 22 h. The reaction mixture was then chromatographed on a Bio-Gel P-10 column with PBS at pH 7.4 as the eluting buffer. The void volume peak was collected and further chromatographed by HPLC on a TSK g3000sw column (LKB Instruments, 7.5 × 600 mm) with an aqueous elution buffer composed of 150 mM NaCl and 15 mM phosphate at pH 7 and a flow rate of 0.8 mL/min. The labeled antibody was collected, dialyzed against PBS (pH 7.4), and stored at −20 °C. The purity of the concentrated sample was assessed by gel permeation chromatography with the TSK g3000sw column; a single peak with the same retention time as the unlabeled antibody was observed, and no free label was present in the sample.

KE-2 IgG was also labeled with DTAF. Conditions used were 9 mol of DTAF/IgG in PBS at pH 9 for 2 h at room temperature. Separation and purification procedures were similar to those used for crabescein labeling.

The number of fluorophores per IgG was determined by measuring the absorbances at 496 and 280 nm and taking their ratio with an extinction coefficient of 8.1×10^4 M^{-1} at 496 nm for crabescein and an optical density of 1.4 for 1 mg/mL of the 150-kdalton protein at 280 nm.

Trypsin Fragmentation. In order to ensure that there would be no interchange between crabescein and any incompletely oxidized disulfide bonds of the protein, the IgG labeled with crabescein was exposed to a 10000-fold molar excess of [^{14}C]iodoacetamide (Du Pont) in PBS (pH 7.4) for 30 min at 37 °C, followed by the addition of cold acetamide (equal amount), and chromatographed on a PD10 (Pharmacia) column with 10% (v/v) acetic acid in the elution buffer. Two column volumes were collected, and the eluant was counted

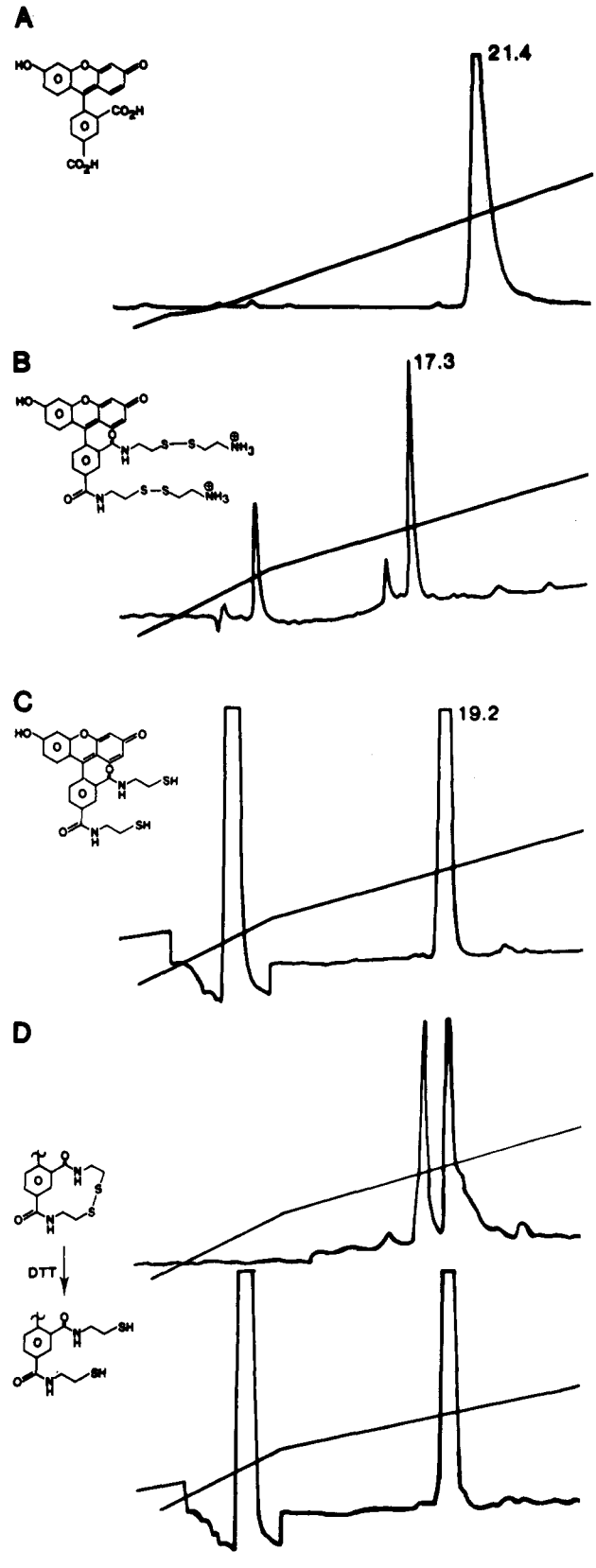

FIGURE 1: Chemical structures and HPLC profiles of (A) starting material, 5′-carboxyfluorescein, (B) procrabescein, and (C) crabescein. Diagonal lines represent a gradient of acetonitrile with the lowest value of 10% and the highest of 90%. (D) Crabescein under oxidative conditions has a tendency to close its claw. The HPLC profile (upper panel) indicates the presence of both reduced (earlier eluting peak) and oxidized forms. This can be reversed, i.e., opened, by treatment with a reducing agent such as DTT as shown in the lower panel.

Table I[a]

derivative	α	τ	χ^2	$\langle r \rangle$	β	ϕ	χ^2
IgG–DTAF	$\alpha_1 = 4.13$	$\tau_1 = 0.27$	1.50	−0.05	$\beta_1 = -0.18$	$\phi_1 = 0.20$	0.91
	$\alpha_2 = 0.87$	$\tau_2 = 1.63$			$\beta_2 = -0.06$	$\phi_2 = 14.3$	
	$\alpha_3 = 0.82$	$\tau_3 = 3.98$					
IgG–crabescein	$\alpha_1 = 0.22$	$\tau_1 = 1.22$	1.50	−0.07	$\beta_1 = -0.09$	$\phi = 26.8$	0.97
	$\alpha_2 = 0.46$	$\tau_2 = 4.41$					

[a] α = preexponential intensity decay factor; τ = fluorescence lifetime (ns); β = preexponential anisotropy decay factor; ϕ = rotational correlation time (ns); χ^2 = chi squared; $\langle r \rangle$ = steady-state anisotropy. Crabescein has a single component lifetime of 4.05 ns and a steady-state anisotropy value of −0.01. λ(excitation) = 313 nm; λ(emission) = 522 nm.

for ^{14}C; no radioactivity above background was detected in the protein peak.

The location of crabescein in the immunoglobulin was determined from the analysis of tryptic IgG fragments. IgG–crabescein (ca. 200 μg) was digested with TPCK-trypsin in a 1% (w/v) ammonium bicarbonate buffer containing 10 mM calcium chloride (pH 8.1) at 37 °C for 3 h in the dark. The ratio of trypsin to antibody by weight was 1:20 with a final volume of 200 μL. The pH of the final digestion solution was adjusted to 9 with a dilute ammonium hydroxide solution. The digestion was terminated by the addition of 100 μL of 0.1% (v/v) trifluoroacetic acid.

The trypsin digestion sample was chromatographed on a Waters Association HPLC equipped with a Gilson fluorescence detector (Model 121) with excitation and emission filters of 496 and 520 nm, respectively, in series with an absorption detector set at 220 nm. A SynChrom C4 column (3.5 mm × 300 mm) was used with water and acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) in the eluting buffer. The gradient was 10% acetonitrile for 10 min followed by a linear gradient to a final composition of 50:50 acetonitrile–water over 40 min at a flow rate of 0.8 mL/min.

The amino acid sequence of the crabescein-containing fragment was determined on an Applied Biosystems gas-phase protein sequencer Model 440.

Fluorescence Measurements. Time-resolved fluorescence decay measurements were made with a mode-locked Yg–Nd laser (Spectra-Physics). The excitation wavelength was 313 nm, and emission was observed at 522 nm. The temperature of the cuvette was 28 °C. The system had a repetition rate of 800 kHz with a full width at half-maximum of 850 ps. One data file was generated with photon collection at the magic angle (54.7°), a second with a polarizer on the emission side perpendicular to the exciting beam, and a third with the polarizer parallel. Data were analyzed by nonlinear least-squares methods (Cundall & Dale, 1983).

Steady-state anisotropies were measured in an SLM 8000 photon counting spectrofluorometer.

Cell Staining. VA-2 cells, a line of SV-40-transformed human fibroblasts, were incubated with the IgG–crabescein or IgG–DTAF at ca. 1 nM for 30 min on ice. The cells were then washed with cold PBS, examined under a fluorescence microscope, and photographed.

Results

Synthesis. Structures of the intermediate and final products are shown in parts B and C of Figure 1, respectively.

The final product (c) contains a claw, was made in Maryland, and was derived from fluorescein; therefore, it was named crabescein. The intermediate product (b) was named procrabescein.

The starting material, procrabescein, and crabescein all exhibited the typical UV–vis spectrum of fluorescein, i.e., a major peak at 496 nm with a shoulder at shorter wavelengths in PBS containing 1% (v/v) ethanol.

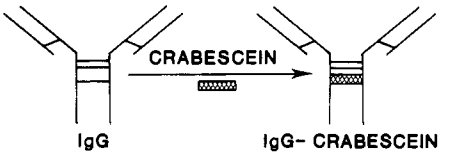

FIGURE 2: Addition of crabescein to IgG_{2a} is across the third (Cys-229) disulfide bond in the hinge region.

The NMR spectra of procrabescein and crabescein in [$^2H_6$]dimethyl sulfoxide ([$^2H_6$]Me_2SO) showed the addition of the methylene groups with split triplets between 2.5 and 3.6 ppm downfield from tetramethylsilane (TMS), slight downfield shifts of the two aromatic protons on the carbons adjacent to the derivatized carbonyls, and two peaks due to the two amide protons. The spectrum of procrabescein also had a broad peak at ca. 6.2 ppm, which integrated for the six amine protons. The spectrum of crabescein showed half the number of methylene protons between 2.5 and 3.6 ppm as procrabescein and the loss of the amine protons; these are consistent with the loss of two aminoethylenemercapto moieties upon reduction. The addition of a peak at 7.2 ppm, which disappeared in the presence of deuterated water, indicated that the exchangeable protons were the sulfhydryls of crabescein. The mass spectrum of crabescein showed a parent molecular ion of 430, which indicated that the parent ion had undergone a rearrangement that resulted in the loss of the two sulfurs.

Incorporation of Dye Molecules. By absorption spectroscopy 0.30–0.42 molecule of crabescein and 1.4 molecules of DTAF were incorporated per IgG.

Trypsin Fragmentation. Only one major fluorescent peak was obtained from the 3-h trypsin digestion of IgG–crabescein by reverse-phase chromatography. The retention time for the major fluorescence-containing peak (ca. 95% of the total fluorescence) was 31 min. After collection, it was further digested with trypsin, and its retention time was not altered. The amino acid sequence of the 31-min peak was analyzed twice with two separate digestion samples by a gas-phase sequencer. Nine Edman cycles were performed, and the amino acid sequence obtained was consistent with the sequence of a tryptic fragment of mouse IgG_{2a} heavy chain at the hinge region (Figure 2) (Bourgois et al., 1974): C-*P*-A-*P*-*N*-*L*-*L*-*G*-*G* (with italized letters indicating the sequence obtained). The levels of phenylhydrodantoin- (PTH-) derived amino acids analyzed ranged from 27 to 7 pmol.

Fluorescence Data (Table I). Fluorescence decay data of crabescein-labeled IgG_{2a} in phosphate-buffered saline are plotted in Figure 3A with the lamp profile curve labeled E, the decay curve collected with the emission polarizer perpendicular to the excitation polarizer labeled H, and the decay curve collected with the emission polarizer parallel to the excitation polarizer labeled V. The top and bottom panels of Figure 3B are plots of the total intensity decay and anisotropy, respectively, as functions of time.

The decay of crabescein's fluorescence in PBS (pH 7.4) was monoexponential with a lifetime of 4.05 ns. Its steady-state

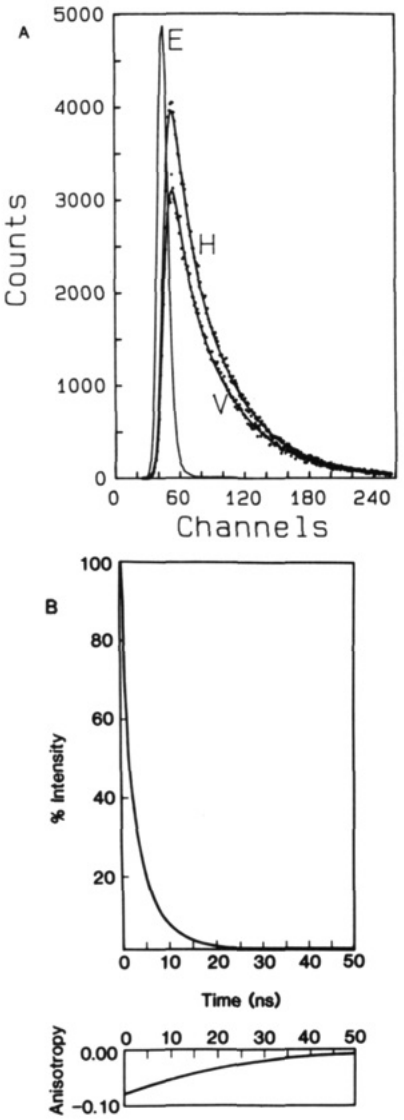

FIGURE 3: Fluorescence decay data of crabescein-labeled IgG_{2a} in phosphate-buffered saline are plotted in (A) with the lamp profile curve labeled E, the decay curve collected with the emission polarizer perpendicular to the excitation polarizer labeled H, and the decay curve collected with the emission polarizer parallel to the excitation polarizer labeled V. The top and bottom panels of (B) are plots of the total intensity decay and anisotropy, respectively, as functions of time.

anisotropy (excitation wavelength = 313 nm; emission wavelength = 522 nm) was −0.01.

DTAF-labeled IgG had three lifetimes: 0.27, 1.63, and 3.98 ns. Its steady-state anisotropy was −0.05, and two rotational correlation times of 0.20 and 14.3 ns were measured. Crabescein-labeled IgG showed two decay constants of 4.41 and 1.22 ns, a steady-state anisotropy of −0.07, and a single rotational correlation time of 26.8 ns.

Cell Staining. VA-2 cells, stained with IgG–crabescein or IgG–DTAF, showed complete outlines of their entire surfaces (Figure 4). All fluorescence was blocked by preincubation with unlabeled antibody.

Discussion

We have designed a fluorescent label that can be rigidly attached to proteins in a gentle and specific manner. The label is targeted to disulfide bonds and achieves its specificity due to the following design elements: (1) disulfides in proteins are relatively limited in number; (2) two attachment sites on the

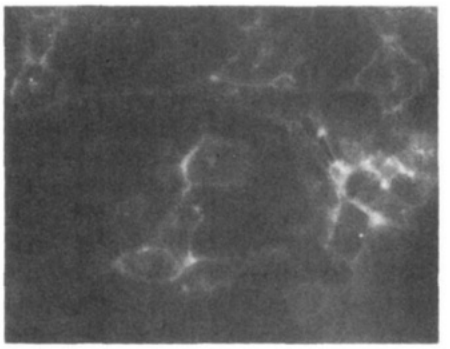

FIGURE 4: Virally transformed (SV-40) human fibroblasts (VA-2 cell line) labeled with IgG–crabescein.

fluorophore prevent free rotation of the label; (3) disulfides are located internal to proteins but are still accessible to solvent molecules as well as to molecules on the size scale of fluorescein; (4) a disulfide bond that is modified by insertion of a fluorophore will retain its tertiary structural stabilizing influence. The latter is in contrast to derivatization by alkylation where one runs the risk of alkylating the nucleophile that may be essential for biologic and/or enzymatic activity of the protein and potentially eliminating the contribution of the disulfide bond to structural stabilization of the protein.

We synthesized crabescein by derivatizing the two carboxyls on the phenyl ring of 5′-carboxyfluorescein with two sulfhydryls. We chose to use an immunoglobulin as the protein to be modified because (1) it has been shown to have a limited number of accessible disulfide bonds and (2) the disulfides of the protein can be reduced without dissociating the heavy and light chains (O'Donnell et al., 1970; Sears et al., 1975; Sutton et al., 1984). Thus, the IgG was unzipped by reduction with DTT, crabescein was inserted into the protein by heteromolecular disulfide bond formation, and the IgG was zipped back up by reoxidation. The amino acid sequence analysis of the fluorescent label containing peptide indicated that the fluorophore was inserted into the hinge region across the disulfide bond closest to the C_2 domain (Figure 2). Crystallographic structural analyses of IgGs have shown that, unlike the region of the interchain disulfide bond in the F_{ab} segment, below the hinge region there is little interaction between heavy chains (Amzel & Poljak, 1979; Davies & Metzger, 1983). Thus, there is a space in this region large enough for a molecule the size of crabescein to enter into and reside in. The observed preferential insertion of crabescein across the disulfide bond closest to the space is quite reasonable.

IgG–DTAF, a derivative in which the macromolecule and fluorophore are linked via one-point attachments, exhibited three decay constants, with none representing more than 56% of the total signal (Table I). It is not possible to say whether the three lifetimes represent three physically distinct sites or are representative of microheterogeneity of ground or excited states (Badea & Brand, 1979; Brand et al., 1985). However, the fact that the species with the lifetime approaching that of free crabescein in solution (ca. 4 ns) represents only 14% of the fluorescing species implicates an interaction between the protein and the probe.

IgG–DTAF exhibits two relatively short (0.20 and 14.3 ns) rotational correlation times (ϕ's). The longer one, 14.3 ns, is in the range (14–18 ns) of the short ϕ that Hanson et al. have ascribed to the V-module flexing at switch peptides in the F_{ab} segment (Hanson et al., 1981, 1985; Hanson, 1985). The four amino termini that exist at the F_{ab} ends are potential labeling sites for DTAF as are other amino groups of the IgG. Thus, the origin of the 14.3 ns is not clear. The subnanosecond

ϕ may report the rapid depolarization due to the autonomous motion of the probe relative to the IgG.

The decay of IgG–crabescein's fluorescence contrasts with that of IgG–DTAF. The intensity decay of the crabescein derivative is best described by two constants with 32% of the fluorescing species exhibiting 1.22 ns and the remaining 68% showng 4.41 ns. The two lifetimes may be a consequence of the asymmetric location of the claw in crabescein such that the fluorophore has the option of inserting in two different directions. Since IgGs have carbohydrate residues near the site at which crabescein attaches, the fluorophore may report on two chemically identical but physically distinct environments.

The amino acid sequence data indicate that the location of crabescein is in the F_c domain. The absence of free sulfhydryls as indicated by the absence of radioactive counts after exposure of the crabescein-labeled IgG to ^{14}C-labeled iodoacetamide in addition to the ability of the antibody to recognize its antigen on the cell surface indicates that we are reporting the rotational correlation time of an intact macromolecule with a possibly slightly perturbed F_c domain. It is certainly possible that an additional longer rotational correlation time(s) similar to those that others have ascribed to the whole IgG do(es) exist (Yguerabide et al., 1970; Brochan et al., 1972; Beale & Feinstein, 1976; Oi et al., 1984; Chan & Cathou, 1977; Lovejoy et al., 1977; Noelken et al., 1965; Romans et al., 1977). However, due to the relatively short fluorescence lifetime of fluorescein, the 28 ns that we observe is near the limit of rotational correlation times observable with a probe with a 4–4.5-ns intensity lifetime (Figure 3B). We are currently extending our synthetic work into longer lived fluorescent and phosphorescent derivatives.

In conclusion, we set out to synthesize a fluorescent compound that would faithfully report the motion of the protein or protein segment to which it was covalently bound. We have reported here the measurement of the rotational correlation time of the F_c domain of a whole IgG to which the reporting group is rigidly bound. Qualitatively and quantitatively, the results are strikingly different from those of the same IgG labeled by a conventional method, i.e., via one-point attachments between the probe and the macromolecule. In the past, others have used tightly bound fluorophores to measure the segmental flexibility of immunoglobulins; however, the price has been the loss of the combining site (Hanson et al., 1981; Yguerabide et al., 1970; Brochan et al., 1972; Oi et al., 1984; Chan & Cathou, 1977; Lovejoy et al., 1977; Noelken et al., 1965; Romans et al., 1977). Crabescein represents a class of labeling reagents with specificity for the F_c segment of immunoglobulins. This class may serve as a useful tool for the study of immunoglobulins' interactions with other macromolecules through the F_c region, e.g., complement, protein A, and cellular F_c receptors.

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