Antibodies for Fluorescent Molecular Rotors

Tomomichi Iwaki, Chikako Torigoe, Masahide Noji, and Mamoru Nakanishi*

Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467, Japan

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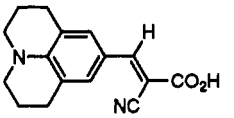
ABSTRACT: We have prepared monoclonal antibodies for the fluorescent molecular rotors 9-(2-carboxy-2-cyanovinyl)julolidine (CCVJ) and 9-(dicyanovinyl)julolidine (DCVJ). Mouse monoclonal antibody (IgG2b) prepared against CCVJ-conjugated bovine serum albumin strongly bound CCVJ and DCVJ. The CCVJ (or DCVJ) binding to IgG and Fab was accompanied by a drastic increase in fluorescence quantum yield, suggesting the restriction of intramolecular rotational relaxation about the donor–acceptor bond of the fluorophores. Nonspecific IgG never changed the quantum yield of the fluorophores. From the Scatchard plots, the association constants of CCVJ to IgG and Fab were 6.8×10^7 and 5.4×10^7 M^{-1}, respectively, and the numbers of moles of CCVJ bound per mole of IgG and Fab were calculated to be 2.0 (±0.1) and 1.0 (±0.05), respectively. The fluorescence spectra of the IgG-bound CCVJ were quite similar to those of Fab-bound CCVJ. The fluorescence lifetimes of the IgG-bound and Fab-bound CCVJ were 388 and 383 ps at 25 °C, respectively. They were 6.3 times as long as the fluorescence lifetime of CCVJ free in solution (62 ps). These results indicated that the drastic increases in quantum yields were due to the decreases of the nonradiative rate constants of the antibody-bound CCVJ, as well as due to the changes of the intrinsic radiative rate constant, and that the nonradiative internal rotations about the donor–acceptor bond of CCVJ were not dependent on the size of the bound antibody molecules. From the nonradiative rate constants, it was suggested that the donor–acceptor bond of the fluorophore may possibly rotate inside the antigen combining site at a rate of 2.4 ns^{-1} at 25 °C. This result is interesting because previous rotational anisotropy studies have examined the molecular motion of the entire protein, not the motion of the probe within a binding site as described here.

Fluorescent molecular rotors are a new class of fluorophores. These molecules are characterized by a charge-transfer-excited singlet state which can rapidly deactivate through internal rotation about the donor–acceptor bond (Law & Loutfy, 1981, 1983; Loutfy, 1981). In a highly constrained environment, the predominant decay pathway is radiative, and we can observe large increases in the fluorescence quantum yield. Thus, these kinds of molecular rotors are thought to be a useful probe to study the assembly of proteins in vitro and in vivo (Kung & Reed, 1989; Furuno et al., 1992) and the dynamic properties of a phospholipid matrix (Kung & Reed, 1986).

Here, we have tried to prepare mouse monoclonal antibodies for the fluorescent molecular rotors, intending to study a new insight concerning the structural basis of antigen recognitions. Binding of fluorescent molecular rotors to IgG molecules was accompanied by a drastic increase in the fluorescence quantum yield, indicating that the intramolecular rotational relaxation of the fluorophore was strikingly restricted. Thus, it seemed that the antibodies for fluorescent molecular rotors would be useful probes to study the dynamic structures of antigen–antibody interactions in solution.

MATERIALS AND METHODS

9-(Dicyanovinyl)julolidine (DCVJ) was prepared from julolidine by a previously reported method (Kuder et al., 1977). 9-(2-Carboxy-2-cyanovinyl)julolidine (CCVJ) was prepared as follows. A mixture of julolidine-9-carboxaldehyde (2 g) and cyanoacetic acid (0.85 g) was dissolved in 10 mL of ethanol. Then, 0.2 mL of a solution of piperidine (0.05 mL) and acetic acid (0.15 mL) was added to the above solution. The solution



CCVJ

was refluxed for 2 h. Dark brown precipitates were recrystallized from ethanol. They melted in the range 198–200 °C.

CCVJ-conjugated bovine serum albumin (BSA) was prepared as follows. *N*,*N*′-Dicyclohexylcarbodiimide (83 mg) in 1 mL of tetrahydrofuran was added to 5 mL of a tetrahydrofuran solution containing *N*-hydroxysuccinimide (46 mg) at 0 °C. The solution was stirred for 2 h at 0 °C and left overnight at room temperature. Then, the solution was filtered to remove precipitated *N*,*N*′-dicyclohexylurea, and the filtrate was dried under reduced pressure. The residue was suspended in 15 mL of dichloromethane, and the suspension was filtered to remove the insoluble ureid. After evaporation, *N*-succinimido-CCVJ was obtained as a reddish powder. Next, *N*-succinimido-CCJV (12.5 mg) and BSA (11 mg) were suspended in a mixture of 4 mL of PBS (0.05 M) and 2 mL of pyridine. The suspension was gently stirred for 48 h at 4 °C. Then, the solution was filtered through a membrane filter (0.45 μm) to remove precipitates, and the filtrate (CCVJ–BSA) was dialyzed at 4 °C. In our present experiments, an average of 9.5 molecules of CCVJ were conjugated to BSA.

Mouse monoclonal antibodies for CCVJ (or DCVJ) were prepared as follows. BALB/c mice were immunized every fourth day for a total of three injections of CCVJ-conjugated BSA emulsified in Freund's complete adjuvant. The dose of antigen per immunization was 45.7 μg. The day after the last injection, lymph node cells were collected and mixed with hypoxanthine–guanine phosphoriboxyl transferase-less my-

* Author to whom correspondence should be addressed. Telephone: (052)836-3411. FAX: (052)836-3414.

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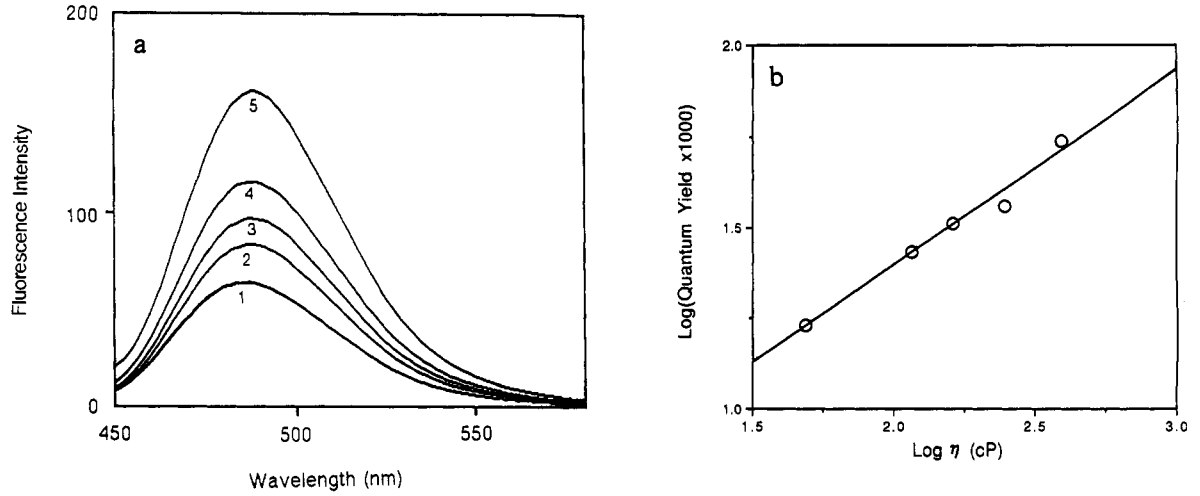

FIGURE 1: Fluorescence spectra of CCVJ in ethylene/glycerol (v/v) mixtures of varying viscosity at 25 °C: (a) mixtures of (1) 7:3, η = 49 cP; (2) 5:5, η = 115 cP; (3) 4:6, η = 163 cP; (4) 3:7, η = 245 cP; (5) 2:8, η = 391 cP. The excitation wavelength was 436 nm. (b) The linear relationship between quantum yield (ϕ_f) and viscosity (η) according to the Förster–Hoffmann expression (Förster & Hoffmann, 1971; Kung & Reed, 1989). Here, the correlation coefficient was 0.980.

eloma PAI cells at a ratio of 2.5:1 in the presence of poly(ethylene glycol) 4000 (Merck) for 2 min. The resultant hybridomas were selected by culturing in the medium containing hypoxanthine, aminopterine, and thymidine. CCVJ-specific hybridomas were screened by ELISA using CCVJ-conjugated lysozyme coated plates. Cloned CCVJ-specific hybridomas were obtained by limiting dilution. To ensure absolute clonality, the cloning procedure was conducted three times at 0.3 cell/well, and the antibody production was confirmed in all cell colonies. Class and subclasses were determined by using a mouse monoclonal antibody isotyping kit (Amersham). Monoclonal antibodies (IgG2b) were purified from ascites fluid by protein A affinity column chromatography using an Ampure PA column (Amersham). Fab fragments were obtained from IgG2b by papain digestion. After digestion, the papain was inactivated by iodoacetamide (10 mM). The solution was passed through an Ampure PA column to remove the Fc-containing fragments. The effluent was then extensively dialyzed against PBS and concentrated using a Centricon-10 (Amicon). The purity of Fab was more than 99%, which was confirmed by SDS–PAGE.

Fluorescence spectra were measured by a Shimadzu Model RF5000 fluorescence spectrophotometer (Ohyama et al., 1991). Fluorescence quantum yields were calculated from a comparison of the integrated corrected emission spectra with fluorescein (ϕ_f = 0.85) as a reference. A decay of fluorescence intensity after pulsed excitation was measured by a Hamamatsu Photonics Model C4780 single photon counting apparatus with an N_2 dye laser. The apparatus was equipped with a streak camera (Hamamatsu Photonics, C4334) (Araiso & Koyama, 1989; Saito et al., 1991). The true decay of the sample was obtained by deconvolution (O'Connor et al., 1979; Ghiggino et al., 1981), and the time resolution of the appratus was about 15 ps.

RESULTS

Fluorescence Properties of 9-(2-Carboxy-2-cyanovinyl)-julolidine. Previous studies have shown that 9-(dicyanovinyl)-julolidine (DCVJ) absorbs strongly in the blue region (450 nm) and has a weak green fluorescence emission in aqueous solution. However, unlike the vast majority of the commonly used fluorophores, the fluorescence quantum yield of DCVJ is dependent on the solvent viscosity or, more explicitly, on the restriction placed on the rotation about the donor–acceptor bond (Kung & Reed, 1989). In a highly constrained environment, the predominant decay pathway is radiative and one can observe a large increase in the fluorescence quantum yield (Loutfy & Law, 1980; Cox et al., 1984; Kung & Reed, 1986, 1989).

In this study, we have freshly prepared 9-(2-carboxy-2-cyanovinyl)julolidine (CCVJ), a derivative of DCVJ, to be covalently bounded to bovine serum albumin (BSA). By conjugation of CCVJ to BSA, we can use it as an antigen to prepare the antibody for fluorescent molecular rotors. CCVJ had the properties of a fluorescent molecular rotor similar to DCVJ. The excitation maximum of CCVJ fluorescence was at 433 nm and the emission maximum was at 500 nm in aqueous solution. Its fluorescence intensity was extremely low, as would be expected for completely unrestricted torsional rotation of a molecular rotor (Kung & Reed, 1989). Figure 1 shows the emission spectra of CCVJ in mixtures of ethylene glycol/glycerol of varying viscosity. These fluorescence properties were similar to those of DCVJ described previously (Kung & Reed, 1989). As the viscosity increased, the fluorescence quantum yield of CCVJ (ϕ_f) increased in accordance with the Förster–Hoffmann expression (Förster & Hoffmann, 1971):

$$\log \phi_f = C + x \log \eta \qquad (1)$$

where η is the viscosity, C is a constant, and x is a free volume term that depends on the structural parameters of the fluorophore. Figure 1b shows the linear relationship between quantum yield (ϕ_f) and viscosity (η) according to the Förster–Hoffmann expression described above. The slope of the line is x = 0.6, which agrees well with the value for DCVJ (Kung & Reed, 1986).

Binding of Antibodies to CCVJ. The fluorescence spectra of CCVJ in aqueous solutions of IgG (IgG2b) are shown in Figure 2. The fluorescence intensity of CCVJ in aqueous solution was extremely low, as would be expected for completely unrestricted torsional rotation of the fluorophore. In the presence of IgG, the fluorescence intensity increased dramatically with increasing concentrations of IgG, and it reached a maximum value at the higher concentrations of IgG. This suggested that CCVJ was bound to IgG and that its binding substantially reduced the rotational freedom of CCVJ. Binding profiles of IgG and Fab fragments to CCVJ (or DCVJ) are shown in Figure 3. Nonspecific mouse IgG never changed the fluorescence intensities of CCVJ. These results suggested that the fluorescence intensity changes

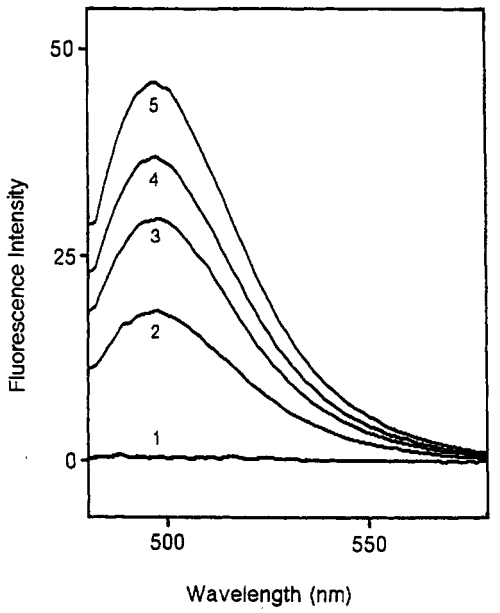

FIGURE 2: Fluorescence spectra of CCVJ in the presence of increasing concentrations of IgG2b at 25 °C. The concentration of CCVJ was 40 nM. The concentration of IgG2b were 0, 12, 25, 50, and 100 μM for curves 1–5, respectively. The excitation wavelength at 460 nm.

observed here were due to the specific binding of CCVJ to the antigen combining sites of IgG. From the Scatchard plots for the binding of CCVJ to IgG of varying concentrations at 25 °C, the association constant was calculated to be 6.8×10^7 M^{-1}. The number of moles of CCVJ bound per mole of IgG was estimated to be 2.0 (±0.1). This was consistent with the number of antigen combining sites of IgG.

The fluorescence spectra of CCVJ in the Fab solution were quite similar to those in the IgG solution, suggesting that the quantum yield of Fab-bound CCVJ was quite similar to that of IgG-bound CCVJ (see Figure 3). From the Scatchard plots for the binding of CCVJ to varying concentrations of Fab at 25 °C, the association constant was calculated to be 5.4×10^7 M^{-1}, and the number of moles of CCVJ bound per mole of Fab was estimated to be 1.0 (±0.05). These results indicated that the intramolecular rotational relaxation of CCVJ in Fab was restricted to an extent similar to that in IgG and that the nonradiative rate constants of CCVJ bound to antibody molecules were not dependent on the molecular size of bound antibody molecules (IgG or Fab).

For comparison, we measured the binding affinity of DCVJ to IgG and Fab (see Figure 3b). The fluorescence intensity of DCVJ was extremely low in aqueous solution, as described previously (Kung & Reed, 1986, 1989); however, it increased dramatically in the presence of IgG. From the Scatchard plots, the association constants of DCVJ to IgG and Fab were 9.3×10^5 and 7.4×10^5 M^{-1}, respectively. The numbers of moles of DCVJ bound per mole of IgG and Fab were 2.0 (±0.1) and 1.0 (±0.05), respectively.

Fluorescence Lifetimes of Antibody-Bound CCVJ. Next, we measured fluorescence lifetimes of IgG- and Fab-bound CCVJ. A time-dependent fluorescence decay of IgG-bound CCVJ is shown in Figure 4. Fluorescence lifetimes were 388 and 383 ps at 25 °C for IgG- and Fab-bound CCVJ, respectively. These values were 6.3 times longer than that of CCVJ free in solution (62 ps).

Then, the fluorescence lifetime (τ) is expressed by

$$1/\tau = k_f + k_{nr} \quad (2)$$

where k_f and k_{nr} are the intrinsic radiative rate constant and the nonradiative rate constant, respectively. And the quantum yield (ϕ_f) is related to the intrinsic radiative rate constant and the nonradiative rate constant by

$$\phi_f = k_f/(k_f + k_{nr}) \quad (3)$$

Thus, from the observed fluorescence lifetimes and the quantum yields, we calculated the values of the intrinsic radiative rate constants (k_f) and the nonradiative rate constants (k_{nr}) of antibody-bound CCVJ and CCVJ free in solution. Calculated values are shown together with the fluorescence lifetimes and quantum yields in Table I.

DISCUSSION

Fluorescence spectroscopy is one of the most sensitive techniques for the analysis of the conformations and dynamic properties of proteins. It has been successfully utilized with fluorescent antigens to study the structural basis of antigen–antibody interactions in solution (Yguerabide et al., 1970; Lovejoy et al., 1977; Hanson et al., 1981; Oi et al., 1984; Stanton et al., 1984; Tan et al., 1990; Kimura et al., 1992). In those experiments, fluorescent antigens were used primarily to detect the changes of the dielectric constants in their environment after the interaction with antibodies, or they were used to measure the fluorescence anisotropy decay rates after being complexed with the antibodies. In this experiment, however, we prepared antibodies for a new group of fluorescent antigens referred to as molecular rotors.

The fluorescent molecular rotors are potentially useful fluorophores whose quantum yields are dependent not on the dielectric constant but rather on the rotational relaxation of the molecules themselves. For these molecules, any process which restricts the rotational relaxation pathway will result in an increase in the fluorescence intensity (Loutfy, 1981, 1986; Loutfy & Law, 1980; Kung & Reed, 1989). As for protein binding, Kung and Reed reported on the fluorescence properties of a fluorescent molecular rotor (DCVJ) in tubulin and in polymerized tubulin assemblies (Kung & Reed, 1989). In their experiments, DCVJ binding to free tubulin was accompanied by an increase in quantum yield, which indicated that the fluorophore had become partially immobilized. Then, the binding stoichiometry was dependent on the concentration of tubulin. Further, when tubulin was polymerized to microtubules or to sheets in the presence of Zn^{2+}, the fluorescence intensity of DCVJ increased dramatically, although the binding stoichiometry still changed (Kung & Reed, 1989). Compared to the above experiments, the antibodies prepared here were able to bind specifically to the fluorescent molecular rotors (CCVJ and DCVJ) with antigen combining sites. Binding constants of CCVJ to the antibodies were much higher than those in the cases of tubulin and bovine calmodulin (Kung & Reed, 1989; Iio et al., 1991). Further, nonspecific mouse IgG never changed the quantum yields of the fluorophores.

In the present experiment, we found that antibody binding decreased the nonradiative rate constant of CCVJ and it increased the intrinsic radiative rate constant of the fluorophore, as shown in Table I. The results suggested that antibody binding decreased the nonradiative internal rotation about the donor–acceptor bond of CCVJ, as well as decreasing the local dielectric constant of the environment around CCVJ.

Then, it has been determined from the fluorescent anisotropy experiments that the rotational motions of the fluorescent antigens were much faster in the complex with Fab than in the complex with IgG (Yguerabide et al., 1970; Hanson et al., 1981; Oi et al., 1984; Osada et al., 1984; Tan et al., 1981). The present results showed, however, that there was no

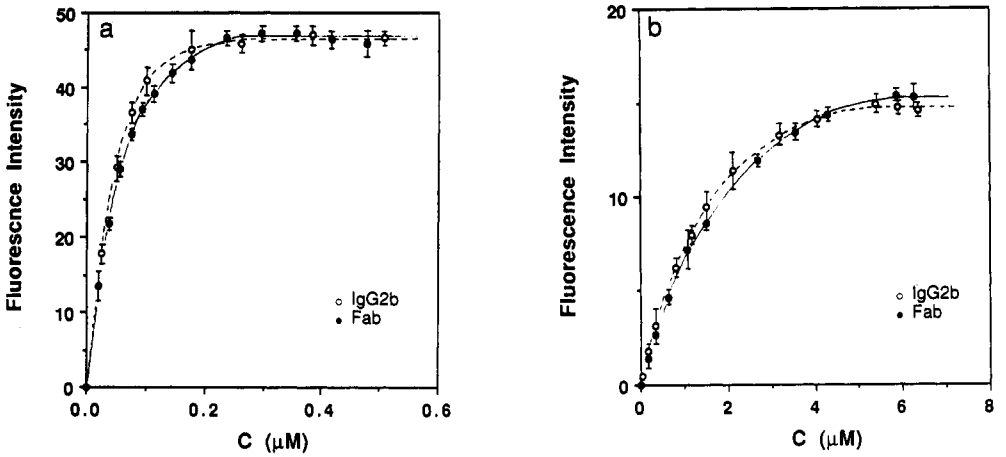

FIGURE 3: Binding profiles of IgG2b (○) and Fab (●) fragments to CCVJ or DCVJ. Antibody bindings to the fluorescent molecular rotors were measured from fluorescence intensity changes at 25 °C. (a) Antibody bindings to CCVJ. Bars are standard deviations. The excitation wavelength was 460 nm, and the emission wavelength was 497 nm. (b) Antibody bindings to DCVJ. Bars are standard deviations. The excitation wavelength was 485 nm, and the emission wavelength was 511 nm.

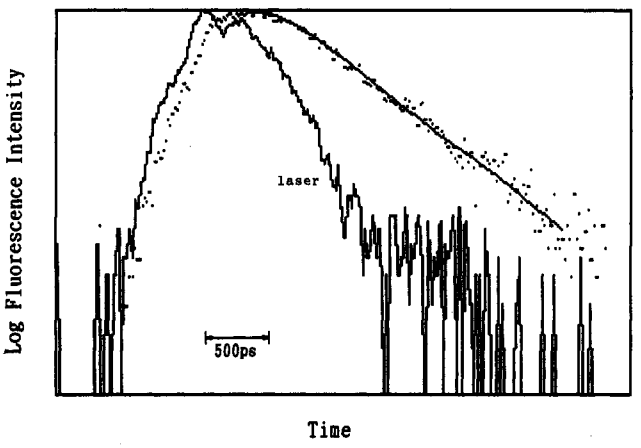

FIGURE 4: Time-dependent fluorescence decay of IgG-bound CCVJ at 25 °C. The excitation wavelength was 460 nm, and the emission wavelength was 490–510 nm.

Table I: Fluorescence Parameters of CCVJ (at 25 °C)[a]

	ϕ_f	lifetime (ps)	k_f (ns^{-1})	k_{nr} (ns^{-1})
free	0.0014 (0.0001)	62 (1.2)	0.023 (0.002)	16.1 (0.3)
IgG-bound	0.062 (0.001)	388 (4)	0.160 (0.003)	2.42 (0.03)
Fab-bound	0.062 (0.001)	388 (4)	0.162 (0.003)	2.46 (0.03)

[a] Numbers in parentheses indicate standard deviations.

difference between IgG-bound CCVJ and Fab-bound CCVJ for the nonradiative rate constants of CCVJ. This suggests that the nonradiative rate constants of the antibody-bound CCVJ do not reflect the rotational motions of antibody molecules themselves, but instead reflect the local rotational motions of the fluorophore in the pocket of antigen combining sites. If so, the rates of the local rotational motion of CCVJ in the antigen combining sites can be approximated by the nonradiative rate constants. They were estimated to be 2.4_2 and 2.4_6 ns^{-1} for IgG-bound CCVJ and Fab-bound CCVJ at 25 °C, respectively. These rate constants were derived from the rates of the rotational movement of the donor–acceptor bond of CCVJ inside the antigen combining sites, because the dissociation rate constants of antigen–antibody complexes were much slower than the rate constants observed here (Lancet & Pecht, 1976; Pecht, 1983).

In conclusion, we have reported the first antibodies directed against so-called molecular rotors and analyzed the properties of CCVJ bound to antigen combining sites. The results are interesting because previous rotational anisotropy studies have examined the molecular motion of the entire protein, not the motion of the probe within a binding site.

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