Metal-Ligand Complexes as a New Class of Long-Lived Fluorophores for Protein Hydrodynamics

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ABSTRACT We describe the use of asymmetric Ru-ligand complexes as a new class of luminescent probes that can be used to measure rotational motions of proteins. These complexes are known to display luminescent lifetimes ranging from 10 to 4000 ns. In this report, we show that the asymmetric complex $Ru(bpy)_2(dcbpy)(PF_6)_2$ displays a high anisotropy value when excited in the long wavelength absorption band. For covalent linkage to proteins, we synthesized the *N*-hydroxy succinimide ester of this metal-ligand complex. To illustrate the usefulness of these probes, we describe the intensity and anisotropy decays of $[Ru(bpy)_2(dcbpy)]$ when covalently linked to human serum albumin, concanavalin A (ConA), human immunoglobulin G (IgG), and Ferritin, and measured in solutions of increased viscosity. These data demonstrate that the probes can be used to measure rotational motions on the 10 ns to 1.5 μs timescale, which so far has been inaccessible using luminescence methods. The present probe $[Ru(bpy)_2(dcbpy)]$ can be regarded as the first of a class of metal-ligand complexes, each with different chemical reactivity and spectral properties, for studies of macromolecular dynamics.

INTRODUCTION

Fluorescence methods are widely used to study the rotational dynamics of proteins, membrane-bound proteins, and other macromolecules (Jameson and Hazlett, 1991; Steiner, 1991; Acuña et al., 1992). The physical basis of these measurements is the polarization or anisotropy of the emitted light when the sample is excited with a vertically polarized light. Information on the rotational motion is available over a time range extending to about 3 times the fluorescence lifetime, after which there is too little signal for accurate anisotropy measurements. Because the lifetimes of typical fluorophores range from 1 to 10 ns, it is difficult to measure rotational correlation times larger than 30 ns. Consequently, it is also difficult to determine the rotational hydrodynamics of larger proteins or membrane-bound proteins.

In an attempt to circumvent this limitation, pyrene and its derivatives have been used as extrinsic labels. Although pyrene derivatives display lifetimes near 100 ns, the initial (time = 0) anisotropy of pyrene is low (typically less than 0.1). In addition, pyrene requires UV excitation, is prone to formation of photo-products, and is thus rather inconvenient as an anisotropy probe. The limitations imposed by the short fluorescence lifetime have been circumvented by the use of phosphorescence anisotropy decays, which have been used to study the rotational dynamics of membrane-bound proteins (Müller et al., 1984; Mühlebach and Cherry, 1985; Bartholdi et al., 1981; Birmachu et al., 1993). Such measurements are based almost exclusively on the triplet probe eosin, which displays a millisecond phosphorescence decay time in the absence of oxygen. Rotational motions have been quantified by transient absorption anisotropy (Mühlebach and Cherry, 1985) and by the time-resolved phosphorescence anisotropy (Bartholdi et al., 1981). However, there are relatively few useful triplet probes. The use of phosphorescence is also inconvenient because of the need to rigorously exclude molecular oxygen, and the low initial phosphorescence anisotropies, typically 0.1 or smaller (Bartholdi et al., 1981; Kawato et al., 1981). Recognition of the limits of present fluorescence and phosphorescence probes has resulted in a combined electron paramagnetic resonance/optical probe to extend the range of accessible rotational correlation times (Cobb et al., 1993). An alternative is the lanthanides, which display long-lived luminescence and are not sensitive to quenching by oxygen. However, these metals are not known to display polarized emission and are thus not useful for studies of rotational motions.

In the present paper, we describe a previously unrecognized opportunity of using the polarized luminescence from metal-ligand complexes to study macromolecular dynamics. Compounds such as tris(2, 2′-bipyridine)ruthenium(II) ($[Ru(bpy)_3]^{2+}$) have been extensively studied for use in solar energy conversion and photochemical catalysis (Juris and Balzani, 1988; Balzani et al., 1986; Seiler et al., 1994; Demas and DeGraff, 1992). These metal-ligand complexes (MLC) display emission from charge-transfer states with decay times ranging from 100 to 4000 ns in fluid solutions with reasonable quantum yields (Demas et al., 1977). Additionally, one can obtain a range of absorption and emission wavelengths, as well as a range of decay times, using complexes of ruthenium, osmium, or rhenium (Kober et al., 1984; Fabian et al., 1980; Sacksteder et al., 1993). However, these

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Abbreviations used: bpy, 2,2′-bipyridine; ConA, Concanavalin A; dcbpy, 4,4′-dicarboxyl-2,2′-bipyridine; HSA, Human serum albumin; IgG, Immunoglobin G, human; MLC, Metal-ligand complex; NHS, *N*-hydroxysuccinimide; TCSPC, time-correlated single photon counting.

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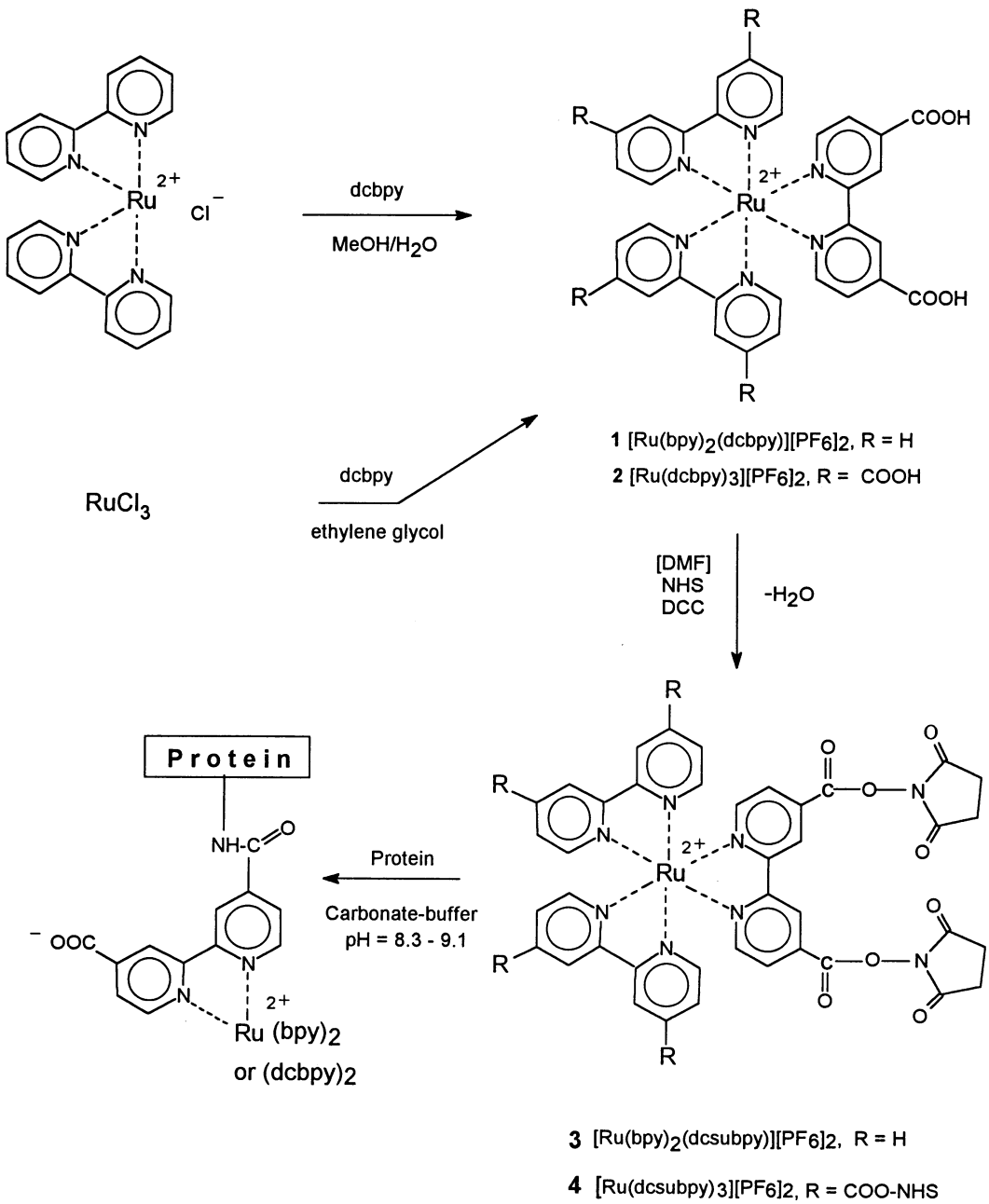
complexes have not been used as anisotropy probes, apparently because of the impression that symmetrical species, such as $[Ru(bpy)_3]^{2+}$, would display low or zero anisotropies. However, we found that a less symmetrical Ru-complex, $[Ru(bpy)_2(dcbpy)]$, displayed high anisotropies in the absence of rotational motions. In the present report, we describe the covalent coupling of this complex to proteins and the measurement of rotational correlation times up to 1.5 μs.

MATERIALS AND METHODS

$RuCl_3$, $Ru(bpy)_2Cl_2$ and $Ru(bpy)_3Cl_2$ were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Chemical synthesis of the NHS-ester of $[Ru(bpy)_2(dcbpy)]^{2+}$ and of the more symmetric complex $[Ru(dcbpy)_3]^{2+}$ is described below and summarized in Scheme 1.

Synthesis of Ru bis(2,2'-bipyridine) (2,2'-bipyridine-4,4'-dicarboxylic acid) bis(hexafluorophosphate) (1)

This synthesis is a modification of that described previously (Kalyanasundaram et al., 1992; Shimidzu et al., 1985). $Ru(bpy)_2Cl_2$ (0.4 g), $NaHCO_3$ (0.4 g), and 2,2'-bipyridine-4,4'-dicarboxylic acid (0.3 g) are heated in MeOH: H_20 = 4:1 for 8–10 h. The solution was cooled in an ice bath for 2 h, and the pH was adjusted with concentrated H_2SO_4 to 4.4. The formed precipitate was filtered, washed with MeOH, the filtrate was treated with 5 g $NaPF_6$ in 25 ml H_2O, then cooled in an ice bath, and the precipitate was collected by filtration. Yield: 0.6 g (77%).



Scheme 1.

Synthesis of Ru tris(2,2'-bipyridine-4,4'-dicarboxylic acid) bis (hexafluorophosphate) (2) (Kalyanasundaram et al., 1992)

$RuCl_3$ (0.1 g), 2,2-bipyridine-4,4'-dicarboxylic acid (3.67 g) were suspended in 15 ml of ethylene glycol and refluxed for 2 h. The solution was cooled to room temperature and filtered. After the addition of 2.5 g $NaPF_6$ in 25 ml H_2O, the pH of the filtrate was adjusted to 1.0 with concentrated H_2SO_4 and the solution was cooled for a few hours. The precipitate was collected and resuspended in MeOH filtered and dried over P_4O_{10}. Yield: 0.38 g (68%).

Synthesis of the NHS esters (3) and (4) and protein labeling

Ru tris(2,2'-bipyridine-4,4'-dicarboxylic acid) *N*-hydroxysuccinimide ester (4). O.46 g of DCC and 0.238 g of *N*-hydroxysuccinimide were dissolved in 3 ml of DMF with stirring and cooled in an ice bath. A solution of 0.38 g of Ru tris(2,2'-bipyridine-4,4'-dicarboxylic acid) (2) was added, and the mixture was stirred for a few hours. The formed precipitate was removed by filtration through a syringe filter, and the filtrate containing the active Ru-complex was used for labeling the substrates. The NHS-ester (3) was prepared in analogy to (4).

The proteins HSA, IgG, ConA, and Ferritin were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The proteins (10 mg portions) were labeled by adding a 100-fold molar excess of the Ru-NHS ester in 50 μl of DMF to 1 ml of stirred protein solution (0.2 M carbonate buffer, pH 8.3–9.1), followed by a 2–6 h incubation and purification of the labeled protein by gel filtration chromatography on Sephadex G-25 or G-50, using 0.1 M PBS, pH 7.2.

Fluorescence intensity and anisotropy decays were measured by time-correlated single photon counting (TCSPC). The primary light source was a cavity-dumped (1 MHz) pyridine 1 dye laser, frequency-doubled to 360 nm. This dye laser was pumped by a mode-locked Nd:YAG laser. The 360 nm output was not used for excitation of the Ru-complex because of the low anisotropy at this excitation wavelength. Hence, we used the 360 nm laser pulses to illuminate a nearly saturated solution of perylene in cyclohexane and a 483 nm interference filter to isolate the perylene emission, which was then used to excite the Ru-complexes. The approximate 5 ns decay time of the "perylene lamp" was easily short enough for the 200–500 ns decay times displayed by our samples. Detection of the emission was accomplished with a Hamamatsu R2809 microchannel plate (MCP) PMT and the usual electronics for TCSPC (Birch and Imhof, 1991).

The time-resolved intensity decays ($I(t)$) were fit to the single- and double-exponential models,

$$I(t) = \sum_{i=1}^{2} \alpha_i \exp\left(-\frac{t}{\tau_i}\right) \quad (1)$$

where α_i are the pre-exponential factors and τ_i the decay times using software from IBH Software (Edinburgh, Scotland). The lamp function was taken as the response observed from a scattering solution at 483 nm illuminated with the perylene "lamp."

The time-resolved anisotropy decays were obtained by measuring the time-dependent decays of the vertically ($I(t)$) and horizontally ($I(t)$) components of the emission

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)}. \quad (2)$$

These data were fit to a single and double correlation time model, again using IBH software.

$$r(t) = \sum_{i=1}^{2} r_{0i} \exp\left(-\frac{t}{\theta_i}\right) \quad (3)$$

where r_{0i} are the amplitudes and θ_i are the rotational correlation times.

Steady-state fluorescence data were obtained using a spectrofluorometer from SLM Instruments, with magic-angle polarizer conditions and an Hamamatsu R-928 detector. The emission spectra are uncorrected.

RESULTS

Absorption spectra of $[Ru(bpy)_2(dcbpy)]$, here called the Ru-complex, are shown in Fig. 1 (*top*). These spectra are normalized to unity to facilitate comparison. The absorption spectra of the Ru-complex depends on pH, in agreement with previous reports that show pK_a values of 2.65 and 0.5 for the carboxy groups (Shimidzu et al., 1985). At pH 7, the net charge on the complex is expected to be zero, with two positive charges on the Ru and two negative charges from the two dcbpy ligands. The long-wavelength absorption spectra of the Ru-labeled proteins are similar, and appear to be intermediate to those observed for the Ru-complex at pH 7 and 0.1. It is interesting to note that the absorption spectrum of $[Ru(bpy)_2(dcbpy)]$ at pH 7 is intermediate between that found for $[Ru(bpy)_3]^{2+}$ and $[Ru(dcbpy)_3]^{4-}$ (Fig. 1, *bottom*).

Emission spectra of $[Ru(bpy)_2(dcbpy)]$ in aqueous solution are shown in Fig. 2. The emission spectra of the Ru-complex is comparable with that observed for $[Ru(dcbpy)_3]^{4-}$ with small red-shift (5 nm). This suggests that the spectral properties of the Ru-complex are determined by the presence of a single dcbpy ligand. Consequently, the

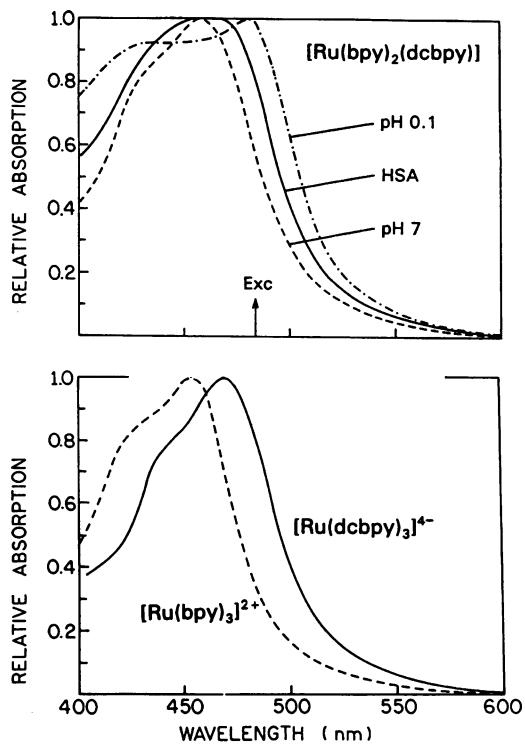


FIGURE 1 (*top*) Absorption spectra of $[Ru(bpy)_2(dcbpy)]$ at pH 0.1 (- - - -) ϵ_{482} = 9870 M^{-1} cm^{-1}, 7.0 (- - - -), ϵ_{460} = 12,000 M^{-1} cm^{-1} (Shimidzu et al., 1985), and when conjugated to HSA, pH 7 (——). Similar spectra were found for the other protein conjugates. (*bottom*) Absorption spectra of $[Ru(bpy)_3]^{2+}$ (- - - -) ϵ_{455} = 14,500 M^{-1} cm^{-1} (Kalyanasundaram et al., 1992), and $[Ru(dcbpy)_3]^{4-}$ (——) ϵ_{470} = 18,000 M^{-1} cm^{-1} (Kalyanasundaram et al., 1992), at pH 7.0.

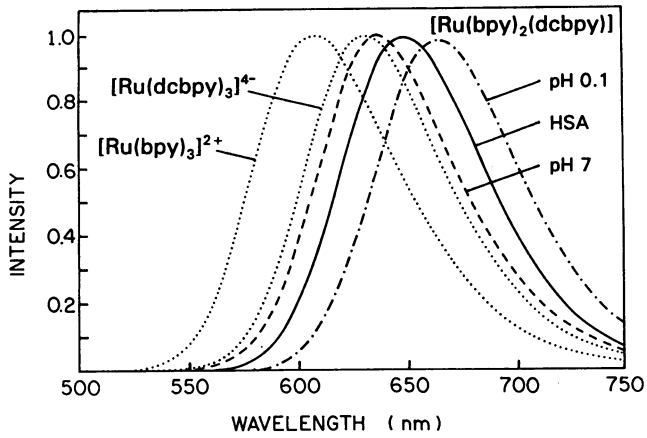


FIGURE 2 Emission spectra of $[Ru(bpy)_3]^{2+}$ and $[Ru(dcbpy)_3]^{4-}$ at pH 7.0 and $[Ru(bpy)_2(dcbpy)]$ at pH 0.1, 7.0 and when conjugated to HSA. Similar emission spectra were found for the other protein conjugates.

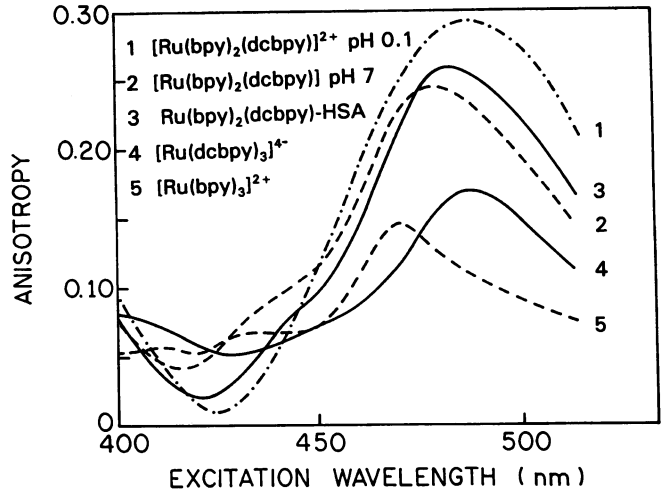


FIGURE 3 Excitation anisotropy spectra of the metal-ligand complexes in glycerol/water (9:1,v/v) at −55°C. The emission wavelength was 650 nm, except for $[Ru(bpy)_3]^{2+}$ where we used 600 nm, with bandpass 8 nm.

anisotropy may be higher than for a more symmetrical complex like $[Ru(bpy)_3]^{2+}$, because the excited state may be localized between the metal and the single dcbpy ligand, rather than being delocalized among three bpy ligands. The emission spectra of the Ru-labeled proteins are similar and also appear to be intermediate to that observed for Ru-complex at pH 7 and 0.1. (Fig. 2). Similar spectra and quantum yields were found for all of the labeled proteins. A somewhat lower quantum yield was found for labeled Ferritin, which is probably because of the long-wavelength absorption of Ferritin and the possibility of Förster and/or Dexter transfer from the Ru to the protein.

We also investigated the effect of dissolved oxygen on the quantum yields. In the absence of oxygen, air equilibrated and oxygen equilibrated buffer solutions, the relative fluorescent intensities were 1, 0.77, 0.44, and 1, 0.89, 0.65, for $[Ru(bpy)_2(dcbpy)]$ and Ru-HSA, respectively. Although this probe is sensitive to dissolved oxygen, the sensitivity is modest and will not require elimination of oxygen to observe the emission from proteins labeled with the Ru complex.

We examined the steady-state excitation anisotropy spectra for $[Ru(bpy)_3]^{2+}$, $[Ru(dcbpy)_3]^{4-}$, $[Ru(bpy)_2(dcbpy)]$ (pH 7), $[Ru(bpy)_2(dcbpy)]^{2+}$ (pH 0.1), and for labeled HSA (Fig. 3), in vitrified solution where rotational diffusion does not occur during the excited-state lifetime. At −55°C the 90% glycerol solution formed a clear glass. Importantly, the asymmetric complex $[Ru(bpy)_2(dcbpy)]$ and its protein conjugates display anisotropies from 0.25 to 0.3 for excitation near 480–490 nm. In contrast, the anisotropy spectra of $[Ru(bpy)_3]^{2+}$ and $[Ru(dcbpy)_3]^{4-}$ display considerably smaller values at all excitation wavelengths. Evidently, the presence of a nonidentical ligand is important for obtaining a useful anisotropy probe. The high anisotropy may be the result of localized electron transfer to the dcbpy ligand (Kalyanasundaram et al., 1992; Shimidzu et al., 1985).

We examined the steady-state anisotropy of the labeled proteins, and of the Ru-complex, over a range of temperatures and/or viscosities (Fig. 4). The solvent was 60%

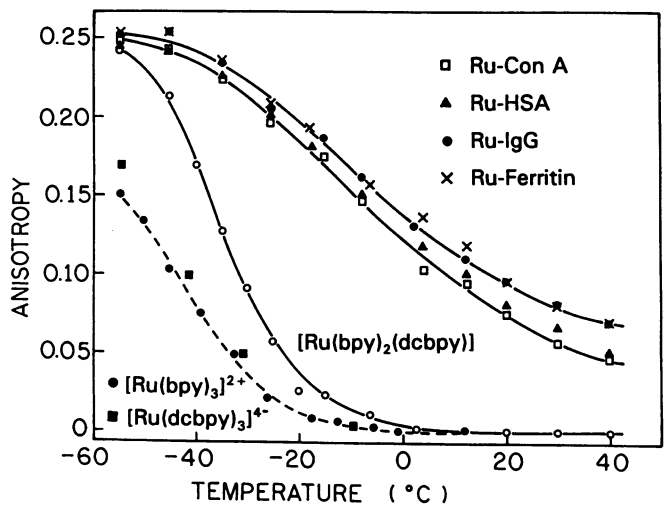


FIGURE 4 Temperature-dependent emission anisotropy of the metal-ligand complexes and protein conjugates in glycerol/water (6:4, v/v). Excitation wavelength was 483 ± 4 nm; emission wavelength was 650 nm and for $[Ru(bpy)_3]^{2+}$ 600 nm, with bandpass 8 nm.

glycerol/40% buffer (v/v), which formed a highly viscous solution glass at −55°C. At low temperatures (−55°C), the anisotropies are nearly identical for the Ru-complex and for the Ru-labeled proteins. The anisotropy value is about 0.25, which is close to the value of 0.28 obtained at −70°C (Ferguson et al., 1989). In contrast, the steady-state anisotropies of $[Ru(bpy)_3]^{2+}$ and $[Ru(dcbpy)_3]^{4-}$ remain low at all temperatures. The low anisotropy of $[Ru(bpy)_3]^{2+}$, obtained at −55°C, is in agreement with other temperature-dependent investigations (Carlin and DeArmond, 1985).

For the Ru-complex and the Ru-labeled proteins, the temperature-dependent anisotropies indicate that the anisotropies are sensitive to rotational motions (Fig. 4). The steady-state anisotropy of the free Ru-complex decreases rapidly above −50°C, whereas the anisotropies of the Ru-labeled

protein decrease more slowly with temperature, and remain relatively high even at 20°C. The steady-state values are only moderately dependent on the molecular weight: Ferritin = 500,000; IgG = 160,000; ConA = 102,000; HSA = 65,000 Da. As will be shown below, the absence of a strict dependence on molecular weight appears to be a result of the complex anisotropy decays of these labeled proteins. Much of the anisotropy of the Ru-protein complexes is lost by fast motions of the probe in addition to rotational motion of the proteins. Importantly, the anisotropies of the labeled proteins are always larger than that of the Ru-ligand complex (Fig. 4), which indicates that protein hydrodynamics contributes to the anisotropy. The detection of rotational motions using these complexes is not an obvious result. A large number of published reports have suggested that the anisotropy and anisotropy decay of the Ru metal-ligand complexes is caused by intermolecular processes such as randomization of the excited state among the three organic ligands and/or interactions with the solvent that result in localization of the excited state after randomization (Yersin and Braun, 1991; Myrick et al., 1987; Blakley et al., 1988; Ferguson et al., 1985).

The range of measurable correlation times is determined by the lifetime of the excited state. We use TCSPC to determine the luminescence lifetimes of the Ru-complex and the Ru-labeled proteins. The intensity decays were closely approximated by a single decay time (Fig. 5). The decay times of the labeled proteins are comparable with that of the Ru-complex alone under comparable experimental conditions (Table 1). The decay times increased somewhat in the presence of glycerol, and at lower temperatures, but the overall range was only about twofold (250–500 ns). As might be expected, the lifetime of Ru-labeled Ferritin is somewhat smaller than that of the other proteins which, as stated above, is probably because of energy transfer to the long-wavelength absorption of Ferritin. The fact that the lifetimes of the complex are similar in solution and when bound to proteins suggests that they are not being quenched by an electron transfer process with the proteins. Additionally, the lifetimes of the complex appear to be rather independent of the extent of labeling, which indicates minimal interactions between the labels on a given protein molecule. The long lifetime of these labels suggests that the Ru-complex can be used to measure rotation correlation times as long as 1.5 μs, about 3 times the luminescence lifetime of the fluorophore.

TABLE 1 Fluorescence lifetimes of [Ru(bpy)$_2$(dcbpy)] and the labeled proteins

Protein	Buffer pH 7.0, 20°C τ (ns)	60% glycerol 20°C τ (ns)	30% glycerol* 20°C τ (ns)	30% glycerol* 5°C τ (ns)	30% glycerol* −15°C τ (ns)
None‡	375	521	472	459	466
ConA	341	509	416	418	416
HSA	336	467	392	467	485
IgG	348	618	427	472	501
Ferritin	250	424	291	369	373

Excitation 483 nm, emission above 540 nm, (Corning 3–67 filter), air equilibrated.
*% glycerol by volume with buffer.
‡Ru-free refers to Ru(bpy)$_2$(dcbpy).

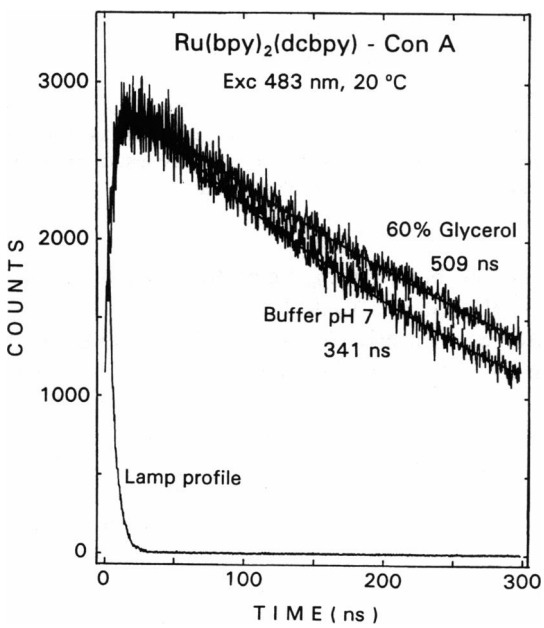


FIGURE 5 Intensity decays of [Ru(bpy)$_2$(dcbpy)] conjugated to ConA. Similar intensity decays were obtained for [Ru(bpy)$_2$(dcbpy)] and conjugated to other proteins (see Table 1).

One may notice that the signal-to-noise ratio is only modest in these data (Fig. 5), which is because of a combination of factors including the inefficient "perylene lamp" and the slow emission rate of the complexes that results in relatively low number of counts per timing channel (from about 1000 to 3000 counts). Nonetheless, these data are adequate for these initial studies to determine the usefulness of these metal-ligand complexes as anisotropy probes. We note that, although the number of photon counts per channel is low, the total number of counts is high, near 10^6, and the decay times are well defined from these data.

To demonstrate that the anisotropy depends on rotational diffusion, we examined the time-dependent anisotropy of free [Ru(bpy)$_2$(dcbpy)] in 60% glycerol-water (v/v) at varying temperatures and viscosities (Fig. 6). At 20°C, the anisotropy decays rapidly with a correlation time near 8 ns. As the temperature is decreased, the anisotropy decays more slowly, with the correlation time increasing to 240 ns at −30°C, and to over 1 μs at −51°C (Fig. 6). Because the lifetime of the Ru-complex is near 500 ns, the intensity has only decayed to about one-third of the initial value at 240 ns. Hence, it should be possible to measure still longer correlation times. At −51°C, the correlation time is longer than 1 μs, with some evidence of a more rapid component near 115 ns. The origin of this shorter component is unknown, and may reflect the role of solvent relaxation in localization of the excited state within the complex. Nonetheless, the near single exponential

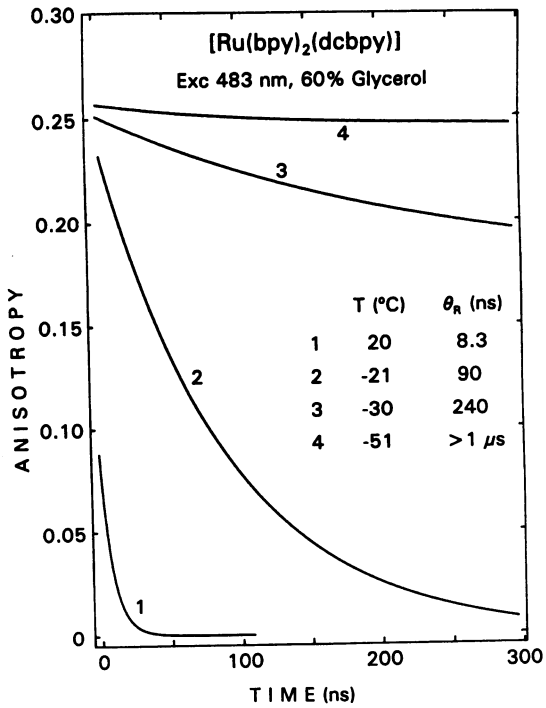


FIGURE 6 Anisotropy decays of free [Ru(bpy)$_2$(dcbpy)] in glycerol/water (60/40, v/v) at the indicated temperatures.

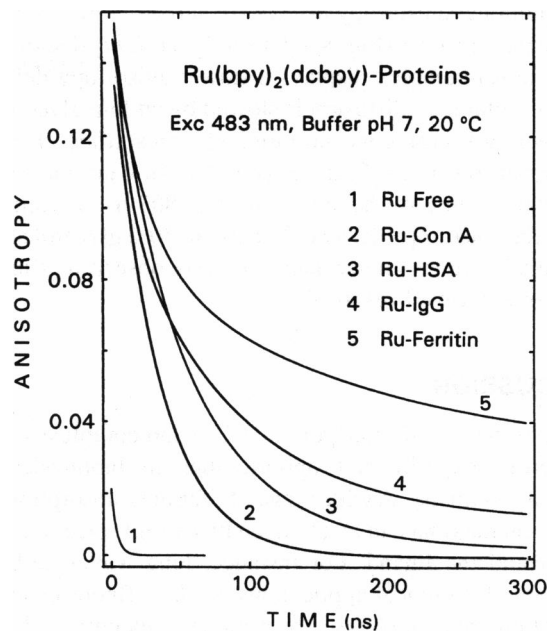


FIGURE 7 Anisotropy decays of [Ru(bpy)$_2$(dcbpy)] in buffer and conjugated to proteins.

anisotropy decays and the apparent activation energy for rotation diffusion near 9.5 kcal/mole (using data from Fig. 6), supports the use of the Ru-complex as a probe of rotational diffusion.

Time-dependent anisotropy decays of the free Ru-complex and the Ru-labeled proteins are shown in Fig. 7. For the Ru-complex alone in buffer at 20°C (i.e., not coupled to proteins), the anisotropy decays within the 5 ns pulse width of the "perylene lamp." In contrast, the anisotropy decays much more slowly for the Ru-labeled proteins. Importantly, the time-dependent decrease in anisotropy becomes slower as the molecular weight of the labeled protein increases. Specifically, Ru-labeled Ferritin displays the slowest anisotropy decay, ConA the most rapid anisotropy decay, and IgG displays an intermediate decay. Although one might expect the anisotropy decay of ConA (M_r 102,000 for the tetramer) to be slower than HSA (M_r 65,000), it is not known whether the ConA subunits dissociate on this timescale, and the shapes of these two proteins may differ. In any event, the data in Fig. 7 demonstrate that the anisotropy decays of the Ru-labeled proteins are sensitive to the size and/or shape of the proteins. In fact, these data have already suggested the presence of a multi-exponential anisotropy decay for IgG, in contrast to the single exponential anisotropy decays of HSA and ConA.These results are reminiscent of the classic report of two relaxation times in IgG as compared with a single relaxation time in the F_{ab} or F_c fragments (Yguerabide et al., 1970).

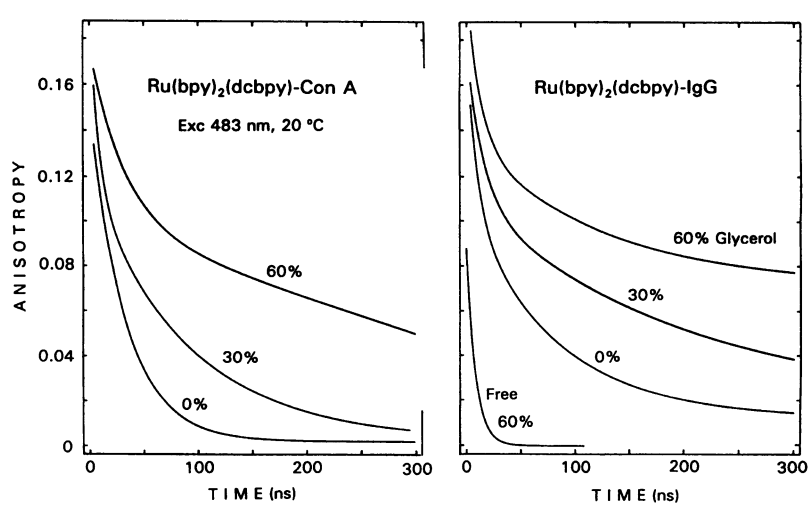


FIGURE 8 Viscosity-dependent anisotropy decays of [Ru(bpy)$_2$(dcbpy)] conjugated to ConA (*left*) and IgG (*right*).

Additional anisotropy decays are shown in Figs. 8 and 9. The data for ConA (Fig. 8, *left*) and IgG (*right*) demonstrate that the Ru-complex displays a slower anisotropy decay as protein rotational diffusion is slowed by adding glycerol. At a given glycerol concentration, the anisotropy decay is slower at lower temperatures (Fig. 9). To date, the longest correlation time we have measured is 807 ns, as estimated from the anisotropy decay of Ferritin in 30% glycerol at 5°C. Correlation times longer than 1 μs were observed (Table 2), but were not well resolved.

TABLE 2 Anisotropy decays of [Ru(bpy)$_2$(dcbpy)] and Ru-labeled proteins

	Buffer pH 7	60% glycerol	30% glycerol		
	20°C	20°C	20°C	5°C	−15°C
Protein	θ (ns)	θ (ns)	θ (ns)	θ (ns)	θ (ns)
None‡	3.9	8.3	4.4	5.8	12.1
HSA	51	139	120	117	73
		15	14	13	*
		212	136	213	
ConA	33	121	90	109	165
		21	15	9	19
		296	96	165	218
IgG	76	120	131	92	167
	9	14	15	38	37
	78	317	200	480	>1 μs
Ferritin	89	133	120	107	112
	24	15	20	28	34
	165	>1 μs	351	807	>1 μs

Excitation 483 nm, emission above 540 nm, (Corning 3–67 filter), air equilibrated. The viscosities at 20°C as estimated to be 1.02, 3.0, and 17 cP for buffer, 30 and 60% glycerol, respectively.
*Does not fit two components.
‡Ru-free refers to Ru(bpy)$_2$(dcbpy).

DISCUSSION

The polarized emission from metal-ligand complexes offers numerous experimental opportunities in biophysics and clinical chemistry. A wide range of lifetimes, absorption, and emission maxima can be obtained by careful selection of the metal and the ligand. For instance, long wavelengths are desirable for clinical applications, such as fluorescence polarization immunoassays. Absorption wavelengths as long as 700 nm can be obtained using osmium (Kober et al., 1984), and lifetimes as long as 100 μs can be obtained using rhenium as the metal in such complexes (Sacksteder et al., 1993). The rhenium complexes also display good quantum yields and high initial anisotropies in aqueous solution. Additional research is needed to identify which of these metal-ligand complexes displays the most favorable spectral properties for a particular application, and to synthesize conjugatable forms of the desired probes.

One disadvantage of the present Ru complex is that its rotational motions are independent of protein rotational diffusion. The anisotropy decays shown above indicate a considerable mobility of the present Ru-complex, which is independent of overall rotational diffusion. If the independent motions can be decreased in amplitude, then a higher fraction of the total anisotropy will be available to detect the hydrodynamics of the proteins. This could be accomplished by using complexes such as the NHS-esters of [Ru(bpy)(dcbpy)$_2$]$^{2-}$ or [Ru(dcbpy)$_3$]$^{4-}$, which would have four or six reactive groups and may bind to proteins by two or more covalent linkages. However, the latter complex displays a lower fundamental anisotropy (Fig. 3), and we do not presently know if this disadvantage can be overcome by multiple covalent linkages. Additionally, such complexes with additional reactive groups may result in protein crosslinking. We are presently investigating the use of other organic ligands that may improve the anisotropy behavior of the metal-ligand complexes. For instance, more hydrophobic organic ligands may enhance noncovalent interactions with the proteins and thus less independent probe motion. Alternatively, the use of other ligands may improve localization of the excited state.

It should also be noted that such long-lived probes can be useful for studies of diffusive processes in a timescale presently not accessible by the usual fluorescence probes. For instance, there is considerable interest in the rates and amplitudes of domain-to-domain motions in proteins, and there

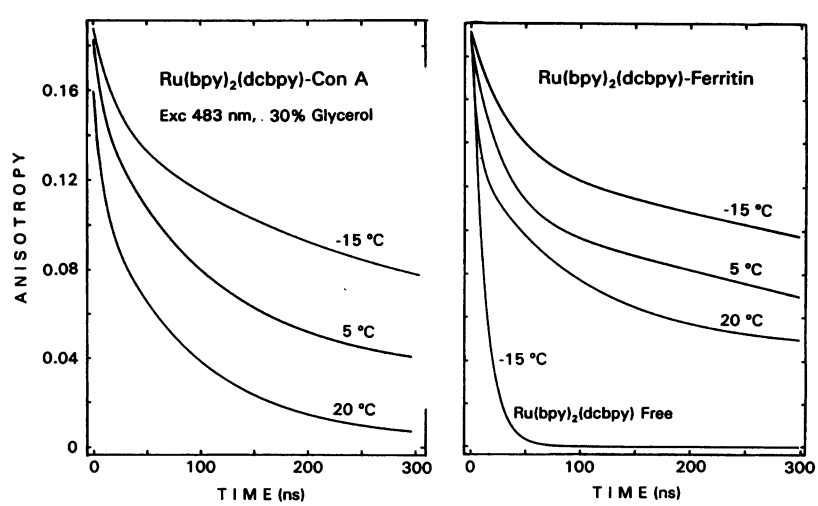


FIGURE 9 Temperature-dependent anisotropy decays of [Ru(bpy)$_2$(dcbpy)] conjugated to HSA (*left*) and Ferritin (*right*) in 30% glycerol.

have been repeated attempts to study such motions by time-resolved fluorescence resonance energy transfer (FRET) (Haran et al., 1992; Eis et al., 1992; Lakowicz et al., 1994). These measurements have been mostly unsuccessful because of the 5–10 ns decay times and the limited extent of inter-domain motions on this timescale. The use of longer-lived MLC emission can allow measurement of these motions. We note that such measurements are not the equivalent of diffusion-enhanced energy transfer using the lanthanide donors, in which the rate of diffusion is not determined, and the data reveal only the distance of closest approach of the donor and acceptor (Thomas et al., 1978; Wensel et al., 1986).

Finally, we note that the MLC can provide considerable information on rotational processes using only steady-state data. It is known that the emission of these complexes can be quenched by a variety of molecules and ions (Hsu and Strauss, 1987; Balzani et al., 1975; Carraway et al., 1991; Lin and Sutin, 1975; Sumi et al., 1985; Ballardini et al., 1987), typically by photo-induced electron transfer to the quencher. The long lifetimes of these complexes suggest that the lifetimes of the labeled macromolecules can be varied over a wide range with modest concentrations of quencher. Steady-state anisotropy measurements, as a function of lifetime or quencher concentrations, can be used to determine the anisotropy decay law of membrane (Lakowicz et al., 1979) and protein-bound (Lakowicz et al., 1983) fluorophores. At present, the use of these complexes as probes is in its infancy, and additional metal-ligand complexes for protein hydrodynamics are yet to be developed.

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