Published in final edited form as:

Anal Biochem. 1997 September 05; 251(2): 241–245. doi:10.1006/abio.1997.2253.

Synthesis and Spectral Characterization of a Thiol-Reactive Long-Lifetime Ru(II) Complex

Ewald Terpetschnig, Jonathan D. Dattelbaum, Henryk Szmecinski, Joseph R. Lakowicz

Center for Fluorescence Spectroscopy, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 725 West Lombard Street, Baltimore, Maryland 21201

Abstract

We report the synthesis and spectral properties of a long-lifetime luminescent Ru complex containing a sulfhydryl-reactive maleimide group, $[\text{Ru}(2,2'\text{-bipyridine})_2(1,10\text{-phenanthroline}-5\text{-maleimide})](\text{PF}_6)_2$. $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$ was covalently linked to human serum albumin, immunoglobulin G, and β -galactosidase. The lifetimes for probe bound to proteins were near 1.1 μs . In the absence of rotational motions, the probe displayed an anisotropy near 0.17 for excitation near 475 nm. Anisotropy decay data were used to determine rotational correlation times of the proteins, which showed local probe motions in addition to overall rotational diffusion. This long-lifetime sulfhydryl-reactive probe can be used to recover microsecond rotational motions and/or domain motions of proteins and/or macromolecular complexes.

Metal–ligand probes with microsecond decay times have numerous potential applications in the biophysical sciences (1–3). Ruthenium (Ru) metal–ligand complexes (MLCs)¹ are known which display long fluorescence lifetimes (4, 5) and are chemically and photochemically stable. The long lifetime increases the sensitivity of measurements using these probes by allowing off-gating of the interfering autofluorescence of biological samples which typically decays in less than 10 ns. In recent publications we demonstrated that certain Ru MLCs display high initial anisotropy in the absence of rotational diffusion (6, 7). This feature combined with long lifetimes has allowed the measurement of rotational correlation times as long as 2 μs and detection of high-molecular-weight analytes in fluorescence polarization immunoassays (7, 8). Additionally, MLC probes have been used to study microsecond dynamics of DNA (9) and for lifetime sensing of pH (10). However, to date, only amine-reactive MLC probes have been described. Because of the smaller number of free sulfhydryl groups in proteins, compared to numerous amino groups, thiol-reactive compounds allow specific labeling at only a few positions in a polypeptide sequence (11–13). Cysteine residues are often placed in proteins by site-directed mutagenesis to provide specific locations for covalent labeling. Sulfhydryl-reactive maleimide derivatives of fluorescein, rhodamine, and pyrene are commercially available and have been used to investigate the structure–function relationships of proteins like the sarcoplasmic reticulum

¹Abbreviations used: HSA, human serum albumin; β -gal, β -galactosidase; IgG, immunoglobulin G; DMF, dimethylformamide; MLC, metal–ligand complex; bpy, 2,2'-bipyridine; phen-NH₂, 1,10-phenanthroline-5-amino; phen-mi, 1,10-phenanthroline-5-maleimide; phen-ITC, 1,10-phenanthroline-5-isothiocyanate; Ru-HSA, HSA covalently modified with $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$; Ru- β -gal, β -gal covalently modified with $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$; Ru-IgG, IgG covalently modified with $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$; Mops, 4-morpholinopropanesulfonic acid.

Ca²⁺-ATPase (11, 12) and Lac repressor (13). Of these probes, pyrene maleimide is the longest-lived derivative, with a lifetime near 100 ns; however, pyrene requires UV excitation, displays a low anisotropy, and is photochemically unstable. Additionally, pyrene cannot be readily modified to result in other probes with longer lifetimes or different spectral characteristics, as is possible with the metal–ligand complexes. We now report the synthesis and spectroscopic properties of a ruthenium (II) metal–ligand complex containing a thiol-reactive maleimide group, [Ru (2,2'-bipyridine)₂(1,10-phenanthroline-5-maleimide)](PF₆)₂ when free in solution and when covalently linked to HSA, β -galactosidase, and immunoglobulin G (IgG).

MATERIALS AND METHODS

Synthesis of [Ru bis(2,2'-bipyridine)(1,10-phenanthroline-5-maleimide)](PF₆)₂ and Protein Labeling

The synthesis of [Ru(bpy)₂(phen-NH₂)](PF₆)₂ was previously described and its spectral properties characterized (7). The orange-colored solid [Ru(bpy)₂(phen-NH₂)](PF₆)₂ (0.45 g, 3.4 mmol) and maleic anhydride (0.1 g, 1.1 mmol) were stirred for 1 h in dimethylformamide (DMF) (Fig. 1). Toluene (3 mL) was added to this solution, and the reaction was refluxed for 4.5 h with a Dean–Stark trap to remove reaction water. Solvents were removed by rotary evaporation, resulting in a dark red residue. The structure of the maleimide was confirmed by its characteristic carbonyl vibration bands in the infrared spectrum at 1770 and 1730 cm⁻¹ (22).

The proteins human serum albumin (65,000 Da) and β -galactosidase (117,000 Da) were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Human IgG was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). IgG was reduced by β -mercaptoethanol for 30 min (14). Each protein was labeled by adding a fivefold molar excess of [Ru(bpy)₂(phen-mi)]²⁺ in 25 μ L of DMF to 750 μ L of a slowly stirring protein solution (5 mg/ml) in 0.2 M phosphate buffer, pH 6.5. Labeling was allowed to occur for 2 h at room temperature. The reaction was stopped by passage through a Sephadex G-25 column, and then the sample was further purified by dialysis overnight using 10 mM Mops, pH 7.4, as buffer.

Spectroscopic Measurements

Absorption spectra were measured on a Perkin–Elmer Lambda 6 spectrophotometer. Steady-state emission measurements were performed on a Aminco-Bowman Model AB2 spectrofluorometer. Polarization effects were eliminated by setting the emission polarizer to 54.7° relative to the vertically polarized excitation light. Excitation polarization spectra were measured at –60°C in glycerol:water (9:1, v/v) using a SLM Model 8000 spectrofluorometer.

Fluorescence intensity and anisotropy decays were measured using frequency domain instrumentation (ISS, Inc.) The polarized output of a cw air-cooled argon ion laser (543-AP Omnichrome) was used to excite the fluorophore. The 488-nm excitation wavelength was amplitude modulated by an electrooptical low-frequency modulator (K2.LF from ISS, Inc.)

The frequency domain intensity decays were analyzed in terms of the multiexponential model

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}, \quad [1]$$

where α_i are the preexponential factors and τ_i are the associated decay times. These values were determined by nonlinear least squares analysis, as described previously (15, 16). The minimized goodness-of-fit parameter χ_R^2 was used to yield the best fit between the data and the calculated values as indicated by

$$\chi_R^2 = \frac{1}{\nu} \sum \left(\frac{\phi_\omega - \phi_{c\omega}}{\delta\phi} \right)^2 + \frac{1}{\nu} \sum \left(\frac{m_\omega - m_{c\omega}}{\delta m} \right)^2 \quad [2]$$

where ν is the number of degrees of freedom and $\delta\phi$ and δm are the uncertainties in the phase and modulation values, respectively. The subscript c indicates calculated values for assumed values of α_i and τ_i . The decay parameters (α_i , τ_i) were used to calculate the average (mean) lifetime, $\bar{\tau}$, and fractional intensities of each component, f_i ,

$$\bar{\tau} = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i$$

and

$$f_i = \alpha_i \tau_i / \sum_i \alpha_i \tau_i. \quad [3]$$

In the frequency domain, anisotropy decays were determined from direct measurements of the phase angle difference () between the polarized components of the emission and the ratio (Λ) of modulated and polarized components. The frequency-domain anisotropy decay were analyzed in terms of the multicorrelation time model

$$r(t) = \sum_j r_{0j} e^{-t/\theta_j}, \quad [4]$$

where r_{0j} is the amplitude of the component displaying a correlation time θ_j (17, 18).

RESULTS

The pathways for the synthesis of Ru-maleimide as well as the covalent attachment of the reactive dye to HSA, β -galactosidase, and IgG are shown in Fig. 1. The maleimido derivative **3** of the Ru complex was prepared by reaction of the Ru(bpy)₂(phen-NH₂)²⁺ **1** with maleic anhydride and subsequent cyclodehydration of the corresponding condensation product **2**. To avoid the reaction with amino-functions in proteins, labeling was performed at a pH of 6.5. At this pH the amino groups in biomolecules are generally protonated and therefore do not react with the maleimide. The dye-to-protein ratio in the HSA conjugate

was estimated to be 0.5:1. As a control experiment using identical reaction procedures, the unreactive amino derivative complex **1** does not remain noncovalently bound to HSA after 24 h of dialysis, indicating that the presence of unreactive free dye will not interfere with our measurements. As a result of this, no further purification of the compound was deemed necessary.

The absorption, emission, and anisotropy spectra of $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$ free in water are shown in Fig. 2. Absorption and emission maxima were found to occur at 467 and 612 nm, respectively. Similar absorption and emission spectra were obtained for the probe covalently linked to proteins. The large Stokes shift is an advantage for high sensitivity detection since scattered stray light is easily eliminated. The excitation anisotropy spectrum (Fig. 2, ---) was measured in the absence of rotational diffusion in glycerol:water (9:1) at -60°C . The limiting anisotropy (r_0) of the free dye was found to be 0.167, which was close to that reported previously for a similar amine-reactive MLC, $\text{Ru}(\text{bpy})_2(\text{phen-ITC})$ ($r_0 = 0.16$) (7).

The emission spectra shown in Fig. 2 show that quenching by oxygen occurs, as is seen from the spectra in the presence and absence of oxygen. Quenching by oxygen results in about a 40% decrease in steady-state luminescence intensity of the free probe in aqueous solution.

Representative frequency-domain intensity decay data for free dye and covalently labeled HSA and IgG are shown in Fig. 3. The frequency response from 20 to 2000 kHz was used to resolve intensity decays of $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$ free and covalently bound to proteins. The detailed analyses of intensity decays in the absence and presence of oxygen (air equilibrated) are summarized in Table 1. The average lifetime of the free probe increased about 30% when bound to each protein in the absence of oxygen. The mean lifetime decrease exhibited in air equilibrated samples was specific for each labeled protein. Ru-HSA (21%) displayed the smallest amount of oxygen quenching, followed by Ru- β -gal (27%) and Ru-IgG (45%).

These observations of oxygen quenching can give information about the microenvironment of the reactive cysteine residue. In aqueous solution the largest value of the oxygen biomolecular quenching constant (k_q) is near $1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ for a completely efficient quencher. The quenching constants, k_q , for Ru-HSA, Ru- β -gal, and Ru-IgG were 0.988×10^9 , 1.46×10^9 , and $2.93 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, respectively. Relative to the free probe, these values represent quenching of 32, 48, and 96% of the maximal rate for Ru-HSA, Ru- β -gal, and Ru-IgG, respectively. Since Ru-HSA displays the smallest amount of quenching, the Cys residue is somewhat shielded from dissolved oxygen; whereas, for Ru-IgG, the labeled Cys residues is mostly exposed to buffer as seen from the observed oxygen quenching constant.

To demonstrate the potential usefulness of $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$ in fluorescence polarization immunoassay or for studies of macromolecular hydrodynamics, the anisotropy decays of $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$ bound to proteins were measured. The frequency domain anisotropy decay data, differential phase angles, and modulated anisotropies are shown in Fig. 4. We suspected that the anisotropy decay data would reflect the protein's molecular weight. Analysis of anisotropy data in Fig. 4 resulted in the rotational correlation times summarized in Table 2. For Ru-HSA, the recovered rotational correlation time of 43 ns was

in good agreement with the predicted and previously reported results (6). In addition, the initial anisotropy of 0.158 was close to that observed in frozen solution of 0.167 (Fig. 2). We concluded that the probe is tightly bound to HSA and reflects the overall rotation of the protein. The results obtained for Ru- β -gal and Ru-IgG were somewhat unexpected. Ru-IgG did not display a longer rotational correlation time than Ru-HSA. The short rotational correlation time of 15–30 ns is poorly resolved because of a very low amplitude (0.034) and the long lifetime of Ru-IgG. This indicates that, unlike the labeling site on HSA, the binding site for [Ru(bpy)₂(phen-mi)]²⁺ on IgG is exposed to buffer where the probe was able to freely rotate.

The anisotropy decay results for Ru- β -gal indicate that the reactive site for the probe is likely to be located on a domain of the protein which undergoes faster rotation than the entire protein. A large fraction of the initial anisotropy is associated with a rotational correlation time of 26 ns, which is significantly less than expected for rotation of the entire protein. Only 15% of r_0 was associated with a long component representative of the entire protein. These data are also consistent with the lifetime measurements, in that oxygen quenching for Ru- β -gal is higher than that for Ru-HSA but less than that for Ru-IgG.

DISCUSSION

We have synthesized and characterized a ruthenium metal–ligand complex containing a maleimide group which is known to be highly specific for reactive cysteine residues in polypeptides. Based on intensity and anisotropy decay data, we have shown that [Ru(bpy)₂(phen-mi)]²⁺ can be used to label free thiol groups in different classes of polypeptides. The position of the cysteine residue(s) within a protein will determine the kind of information which can be obtained from using long-lifetime metal ligand complexes.

Taken together these results provide valuable information into the future application of probes like [Ru(bpy)₂(phen-mi)]²⁺. Potential applications in the study of protein interactions arise from the small number of reactive cysteine residues found in a protein. As mentioned earlier fluorescence polarization immunoassays of high-molecular-weight species will be possible with long-lifetime probes (8, 18). Previously, such assays were limited to low-molecular-weight analytes. In addition, fluorescence resonance energy transfer measurements can be used to investigate intra- or interdomain diffusional processes (20), as well as distance distributions (21) between a well-defined donor located on a cysteine residue and a randomly positioned acceptor molecules. [Ru(bpy)₂(phen-mi)]²⁺ can thus be useful for measuring rotational motions, domain motions, and translational diffusion in protein and membranes.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (RR-08119 and GM-35154), with support for instrumentation from the NIH (RR-10416). J.R.L. also expresses appreciation for support from the Medical Biotechnology Center at the University of Maryland.

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FIG. 1.
Synthetic scheme for $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$.

FIG. 2. Absorption and emission spectra of $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$ in buffer. The dashed line shows the excitation anisotropy spectra at -60°C in glycerol:water (9:1, V/V).

FIG. 3. Representative frequency-domain intensity decays of $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$ when free in solution and covalently linked to HSA and IgG.

FIG. 4. Frequency-domain anisotropy decays of $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$ when covalently linked to HSA, IgG, or β -gal.

TABLE 1

Intensity Decay Analysis of $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$ and When Conjugated to HSA, IgG, and β -Gal^a

Protein	τ_1 (ns)	a_i	f_i	ns	χ_R^2 ^b
Free/Ar	364	0.365	0.195		
	860	0.635	0.805	763	2.9 (29.4) ^c
Free/air	216	0.259	0.126		
	521	0.741	0.874	483	2.1 (17.2)
HSA/Argon	62	0.527	0.066		
	650	0.277	0.360		
	1458	0.195	0.574	1076	1.0 (19; 119)
HSA/air	72	0.481	0.079		
	568	0.357	0.458		
	1261	0.162	.463	850	1.6 (15; 852)
β -Gal/Ar	66	0.442	0.052		
	679	0.380	0.460		
	1533	0.178	0.488	1064	1.6 (16; 582)
β -Gal/air	56	0.448	0.063		
	512	0.429	0.552		
	1248	0.123	0.385	767	3.9 (19; 499)
IgG/Ar	92	0.308	0.038		
	783	0.403	0.422		
	1403	0.289	0.542	1094	1.2 (4; 200)
IgG/air	28	0.461	0.039		
	415	0.253	0.319		
	737	0.286	0.642	607	1.4 (4; 256)

^aMeasurements were performed at 23°C, in the presence (air) and absence (Ar, argon) of dissolved oxygen from the air.^bFor calculation of χ_R^2 , we used $\delta\phi = 0.2$ and $\delta m = 0.007$.^cValues in parenthesis show χ_R^2 for two- and one-component fits.

TABLE 2Anisotropy Decay Analysis of $[\text{Ru}(\text{bpy})_2(\text{phen-mi})]^{2+}$ Conjugated to Proteins, HSA, IgG, and β -Gal^a

Protein	ϕ (ns)	r_{Oi}	Σr_{Oi}	χ^2_{R}
HSA	42.6	0.158	0.158	5.3 ^b
β -Gal	26	0.091		
	498	0.026	0.117	4.4
IgG	28	0.034	0.014	3.0
	17	—	$\langle 0.15 \rangle$	4.1

^aSee Table 1 for additional details.^bThe value of χ was calculated using $\delta\phi = 0.2$ and δm Å 0.007.