Molecular Recognition of a Monoclonal Antibody (AC1106) Cross-Reactive for Derivatives of $Ru(bpy)_3^{2+}$ and $Ru(phen)_3^{2+}$

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Abstract: The characterization of a monoclonal antibody (AC1106) elicited *via* immunization with a $Co(dmbpy)(bpy)_2^{3+}$−methyl viologen hapten (**1**) is described. AC1106 was found cross-reactive for a variety of luminescent ruthenium(II) metal complexes which served as useful probes to investigate the molecular recognition properties of this antibody. AC1106 was found to be specific for methylated derivatives of $Ru(bpy)_3^{2+}$ and $Ru(phen)_3^{2+}$ in the order of $Ru(dmbpy)_3^{2+} > Ru(dmbpy)(bpy)_2^{2+} > Ru(dmphen)_3^{2+} > Ru(bpy)_3^{2+} \gg Ru(phen)_3^{2+}$. The affinities of AC1106 for these metal complexes were found to range from $\geq 5 \times 10^7$ to $\leq 1 \times 10^3$ M^{-1}. When bound (>98%) by AC1106, the luminescence decay traces for the racemic $Ru(dmbpy)_3^{2+}$ and $Ru(dmbpy)(bpy)_2^{2+}$ gave a satisfactory fit to a single-exponential decay process. Furthermore, D_2O/H_2O experiments with $Ru(dmbpy)_3^{2+}$ indicate that AC1106 protects approximately 70% of the antibody-bound $Ru(dmbpy)_3^{2+}$ from excited state deactivation by the solvent. Competition ELISA data indicate that both the metal center and the methyl viologen moiety present in a $Ru(bpy)_3^{2+}$−methyl viologen conjugate ($[Ru(mv^{2+}\text{-}bpy)(bpy)_2]^{4+}$) are important recognition elements for AC1106. Despite the apparent affinity of AC1106 for methyl viologen, no evidence for simultaneous binding of methyl viologen and $Ru(dmbpy)(bpy)_2^{2+}$ inside the binding pocket of AC1106 could be found. Rather, the addition of methyl viologen was found to result in the displacement of AC1106-bound $Ru(dmbpy)(bpy)_2^{2+}$ from the antibody binding site.

Introduction

Antibodies with high affinity and exquisite specificity can be elicited by repeated immunization with a molecule of interest. Antibodies[1] can be considered a unique type of media in that they can be "tailor-made" to bind a desired ligand[2] *via* immunization with an appropriately chosen hapten. The association between antibody and antigen is the result of immunologically tuned, complementary interactions (e.g., charge−charge, dipole−dipole, and π-stacking) between an antibody ligand and amino acid residues in the binding pocket. Large association constants for antibody ligands (up to 10^{12} M^{-1}) and a high degree of selectivity are common.[3,4] Thus, antibodies are useful in the design of both catalytic[5] and recognition based systems[6] where high affinity and specificity are desirable elements.

Of considerable importance in the design of antibody-based systems is a fundamental understanding of the binding properties associated with antibody−antigen interactions. One approach to understanding these properties is through the use of luminescent probes. Both polyclonal and monoclonal antibodies specific for, or elicited by, luminescent molecules such as adriamycin,[7] derivatives of dansyl chloride,[8] 9-(2-carboxy-2-cyanovinyl)julolidine,[9] and fluorescein[10] have been reported. By far, the most extensively studied set of data exists for fluorescein. In general, antibody binding of fluorescein results in fluorescence quenching.[11] Use of this spectroscopic handle, pioneered by the research group of Voss,[12] has made possible a variety of elegant studies that include investigations of hapten dissociation and association rates[13] and kinetic affinity maturation.[14]

One class of luminescent molecules that has gone relatively unexplored as antibody probes is that of ruthenium(II) metal complexes, such as $Ru(bpy)_3^{2+}$ and $Ru(phen)_3^{2+}$ (Figure 1). Several qualities of these metal complexes facilitate their use as probes of antibody response and recognition, including their stability in water,[15] synthetic accessibility to a variety of metal complexes of a well-defined shape,[16] and long-lived excited

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(2) Ligand, as used throughout this paper, has two meanings: It signifies either a small molecule that binds within an antibody binding pocket or a chelate for a metal ion. In addition, "cross-reactive" is an immunological term indicating that the antibody is capable of binding a ligand other than what it was designed for (i.e. the hapten). Cross-reactive is not meant to indicate that a chemical reaction takes place within the antibody binding pocket.

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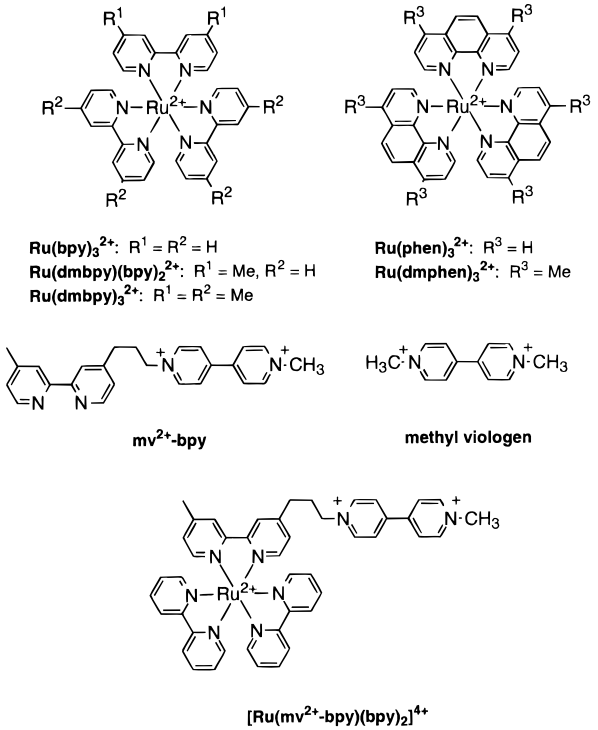

Figure 1. Structures and abbreviations for the compounds used in this study.

states[17] that are sensitive to both solvent[18] and quenchers such as O_2.[19] As demonstrated in this study, when used as model antibody ligands, these compounds can provide important information on the nature of antibody−antigen binding.

The excited state properties of these compounds have led to important applications in the areas of both electron transfer studies and catalysis.[20,21] As a result, the photophysical and molecular recognition properties of this general class of compounds have been studied in a variety of biological and abiological organized media, such as DNA,[22] micelles,[23] clays,[24] polyelectrolytes,[25] and zeolites.[26] Consequently, these systems provide well-studied background for comparison and contrast. Despite the extensive use of these metal complexes, antibody-based studies have been limited to our previous report of a *polyclonal* antibody system that binds Ru(bpy)$_3^{2+}$.[27]

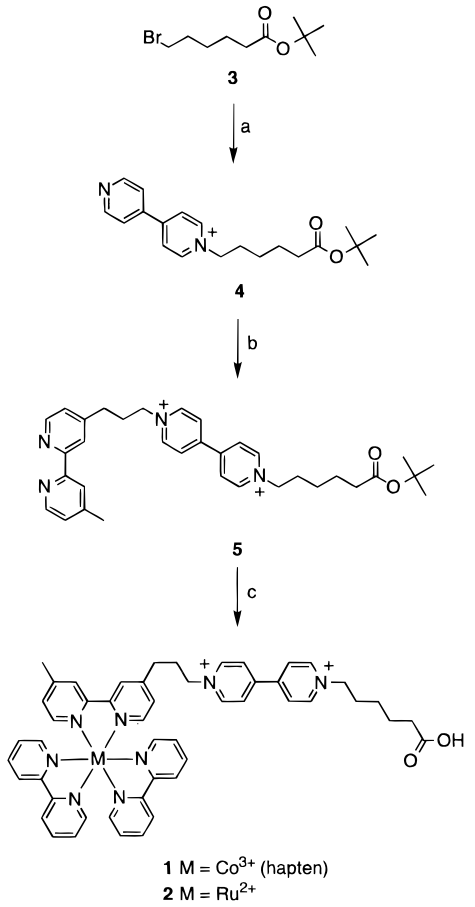

Figure 2. Synthetic scheme for the synthesis of the hapten **1** and the corresponding ruthenium(II) analogue **2**: (a) 4,4′-bipyridine, CH_3CN, reflux (35%); (b) 4-(3-bromopropyl)-4′-methyl-2,2′-bipyridine, CH_3CN, reflux (45%). (c) For **1**: $[Co(bpy)_2]Cl_3 \cdot H_2O$, H_2O then TFA, dioxane (30% from **5**). For **2**: $[Ru(bpy)_2]Cl_2 \cdot 2H_2O$, MeOH, reflux (**6**) then 1:1 H_2O:TFA (67% from **5**).

For the present *monoclonal* antibody study, a Co(dmbpy)-(bpy)$_2^{3+}$−methyl viologen hapten (**1**) was synthesized (Figure 2). Several elements went into the design of this hapten: The kinetically inert cobalt(III) metal center was chosen based on its previously reported stability to the conditions of immunization.[28] In addition, the dimensions of Co(bpy)$_3^{3+}$ roughly approximate those of Ru(bpy)$_3^{2+}$,[29] and we anticipated that an antibody capable of binding the hapten **1** would also be capable of accommodating Ru(bpy)$_3^{2+}$ within the same binding pocket. Finally, methyl viologen was tethered within close proximity

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of the Co(dmbpy)$(bpy)_2^{3+}$ metal center in an attempt to elicit a binding site capable of complexing Ru$(bpy)_3^{2+}$ and methyl viologen simultaneously. Following immunization with **1**, a murine monoclonal antibody (AC1106) cross-reactive for both **1** and **2** was isolated. In this study, AC1106 is shown to exhibit a highly structure dependent binding mode for derivatives of Ru$(bpy)_3^{2+}$ and Ru$(phen)_3^{2+}$.

Results and Discussion

Steady-State Luminescence Studies. Steady-state luminescence spectroscopy proved to be a useful tool in detecting the specific binding of various luminescent ruthenium(II) metal complexes to the monoclonal antibody AC1106. Shown in Figure 3a is the integrated steady-state emission intensity recorded for a fixed amount of Ru(dmbpy)$(bpy)_2^{2+}$ in the presence of increasing amounts of AC1106. It can be seen that the addition of AC1106 to a solution of Ru(dmbpy)$(bpy)_2^{2+}$ results in enhanced luminescence from the metal complex. As important controls, it was noted that upon titration of Ru(dmbpy)$(bpy)_2^{2+}$ with both bovine serum albumin (BSA) and nonspecific IgG antibody there was no significant enhancement in the emission intensity. These control studies, therefore, confirm that the luminescence enhancement seen upon titration with AC1106 is the result of a binding event within the antibody pocket and does not arise from nonspecific binding to a protein surface. Shown for comparison in Figure 4 are the emission spectra at the beginning and end of the titration with AC1106: There is a slight blue shift observed for the emission maximum of the AC1106-Ru(dmbpy)$(bpy)_2^{2+}$ complex relative to free Ru(dmbpy)$(bpy)_2^{2+}$. In addition, the change in the total integrated emission intensity upon binding to the antibody to free metal complex is a factor of 2.3, a value well above the experimental error.

When these same experiments were performed with Ru(dmbpy)$_3^{2+}$, similar results were obtained. Shown in Figure 3b is the integrated steady-state emission intensity measured for a fixed amount of Ru(dmbpy)$_3^{2+}$ in the presence of increasing amounts of AC1106. As with Ru(dmbpy)$(bpy)_2^{2+}$, a significant enhancement in emission intensity was observed upon titration with AC1106 but no such enhancement was observed when the antibody was replaced with either BSA or nonspecific antibody. Again, there is a slight blueshift in the emission maximum upon binding (see supporting information). Here, the change in the total integrated emission intensity of the antibody bound to free metal complex is a factor of 2.6.

The amplification in emission intensity noted upon binding of these metal complexes to AC1106 provides a convenient way to derive the corresponding association constants.[30] Thus, for Ru(dmbpy)$(bpy)_2^{2+}$, the K_a was determined to be 1.2×10^7 M^{-1} (Figure 3a) whereas for Ru(dmbpy)$_3^{2+}$ a lower limit for the K_a of 5×10^7 M^{-1} was set based on the linear nature of this titration (see Figure 3b). Interestingly, an association constant for AC1106 binding to Ru$(bpy)_3^{2+}$ could not be determined using this method since there was no amplification of the luminescence in this case (Figure 3b). This result serves to indicate that the affinity of AC1106 for Ru$(bpy)_3^{2+}$ is relatively low.

The steady state luminescence data demonstrate that the presence of methyl groups in the 4 and 4′ positions of the 2,2-bipyridine ligands in Ru(dmbpy)$(bpy)_2^{2+}$ and Ru(dmbpy)$_3^{2+}$ plays a crucial role in recognition by AC1106. Interestingly, two types of metal ligands are present in the hapten **1** used to elicit the immune response that gave rise to AC1106, namely, unsubstituted 2,2′-bipyridine groups and **5**. Because **5** is derived from 4,4′-dimethyl-2,2′-bipyridine, the apparent "methyl-specific" mode of binding might be taken to indicate that the immunological process that produced AC1106 gave particular emphasis to the substituted ligand of the hapten. This may be because the greater complexity of this latter ligand provides for more specific molecular recognition.

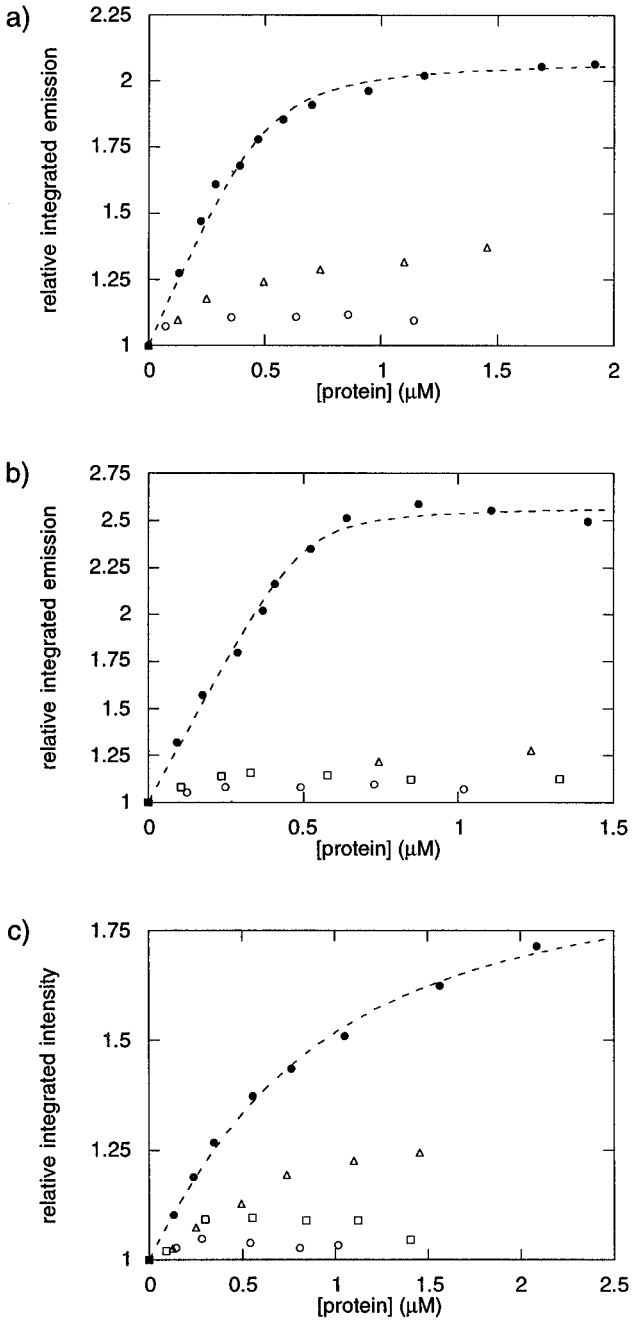

Figure 3. (a) Steady-state luminescence titration of 1×10^{-6} M Ru(dmbpy)$(bpy)_2^{2+}$ with AC1106 (●), BSA (△), and nonspecific IgG antibody (○). Shown is an interpolative fit for the K_a of AC1106 binding to Ru(dmbpy)$(bpy)_2^{2+}$ of 1.2×10^7 M^{-1}. (b) Steady-state luminescence titration of 1×10^{-6} M Ru(dmbpy)$_3^{2+}$ with AC1106 (●), BSA (△), and nonspecific IgG antibody (○). Shown is an interpolative fit for the K_a of AC1106 binding to Ru(dmbpy)$_3^{2+}$ of 3.4×10^7 M^{-1}. Also shown is the titration of Ru$(bpy)_3^{2+}$ with AC1106 (□). (c) Steady-state luminescence titration of 1×10^{-6} M Ru(dmphen)$_3^{2+}$ with AC1106 (●), BSA (△), and nonspecific IgG antibody (○). Also shown is the titration of Ru$(phen)_3^{2+}$ with AC1106 (□). Shown is an interpolative fit for the K_a of AC1106 binding to Ru(dmphen)$_3^{2+}$ of 8.4×10^5 M^{-1}.

(30) The data could best be fit by using a 1:1 ratio of antibody pockets to bound metal complex, so this mode of binding was assumed under the conditions of the experiments presented in this paper.

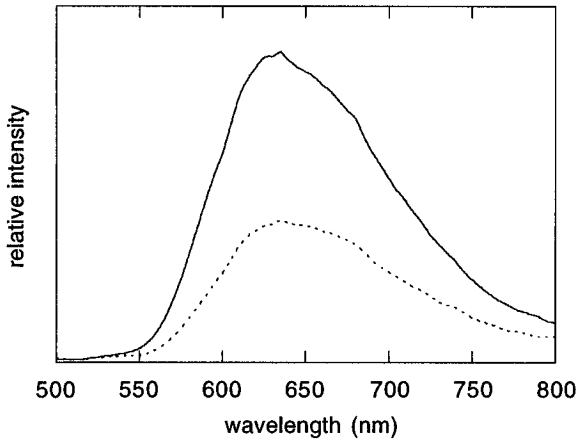

Figure 4. Emission spectra at the beginning (- - -) and end (—) of the titration of Ru(dmbpy)$(bpy)_2^{2+}$ with AC1106 shown in Figure 3a.

Alternatively, or in conjuction with this idea, is that increased hydrophobicity of the more highly methyl-substituted metal complexes leads to a higher affinity for AC1106. Indeed, strict methyl-group recognition could not be used as a rationalization for the increased affinity of Ru(dmbpy)$_3^{2+}$ over Ru(dmbpy)-$(bpy)_2^{2+}$: Because the antibody binding pocket of AC1106 was induced by **1**, this pocket should be shape-selective for this hapten. As a result, one would not expect the four "additional" methyl groups present on Ru(dmbpy)$_3^{2+}$ to afford favorable protein contacts with AC1106 when only two methyl groups are present in the hapten. However, if the binding pocket of AC1106 possesses considerable hydrophobic character, it may exert a preference for more hydrophobic metal complexes.[31]

To probe the effect that shape selectivity has on binding, experiments were carried out with Ru(dmphen)$_3^{2+}$. This metal complex, by virtue of its bulkier 1,10-phenanthroline-derived ligands, is a poor steric mimic of the Co$(bpy)_3^{3+}$-based hapten when compared to its 2,2′-bipyridine-derived congener, Ru-(dmbpy)$_3^{2+}$. Shown in Figure 3c is the integrated steady-state emission intensity recorded for a fixed amount of Ru-(dmphen)$_3^{2+}$ in the presence of increasing amounts of AC1106. As with the 2,2′-bipyridine-derived ruthenium(II) metal complexes, a significant emission enhancement can be seen upon titration with AC1106 whereas BSA or nonspecific antibody exhibit no such effect. Using this enhancement as a means to calculate the association constant for interaction between AC1106 and the metal complex yields a value of 8.4×10^5 M^{-1}. The 100-fold lowered affinity for Ru(dmphen)$_3^{2+}$ versus Ru(dmbpy)$_3^{2+}$ can be explained by the relatively poor steric fit of the former metal complex within the binding pocket of AC1106. Interestingly, as in the 2,2′-bipyridine series, no significant enhancement of the emission for the methyl-free Ru-(phen)$_3^{2+}$ was seen upon titration with AC1106 (Figure 3c). This observation again emphasizes that methylated ligands are crucial components for recognition of metal complexes by AC1106.

Time-Resolved Luminescence Studies. Time-resolved luminescence spectroscopy was also used to study the interaction of AC1106 with these same derivatives of Ru(bpy)$_3^{2+}$ and Ru-(phen)$_3^{2+}$. In the absence of antibody, the luminescence profiles recorded for the metal complexes could be analyzed satisfactorily in terms of a single exponential, in each case the derived lifetimes depending on the concentration of dissolved oxygen but being independent of laser power and chromophore concentration. When AC1106 was titrated into a solution of Ru-(dmbpy)$(bpy)_2^{2+}$ or Ru(dmbpy)$_3^{2+}$, a longer lived component appeared in the luminescence decay profiles, in addition to the component characteristic of free metal complex. This new component was assigned to the antibody-bound metal complex, in line with the results of the steady-state luminescence studies. At higher concentrations of AC1106, the luminescent decay traces of both metal complexes gave a satisfactory fit to a single-exponential decay process in both air- and N_2-saturated solution (Figure 5). The excited-state lifetimes recorded under such conditions, which are significantly longer than those recorded in the complete absence of antibody, are assigned to the antibody-bound metal complex.

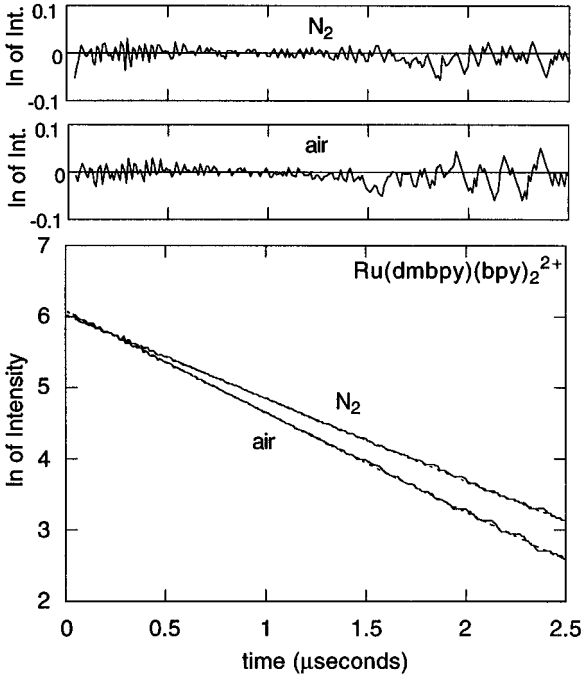

Figure 5. Semilogarithmic plot of the luminescence decay trace of Ru(dmbpy)$(bpy)_2^{2+}$ (1.6×10^{-5} M) in the presence of AC1106 (4.9×10^{-5} M in Ab binding sites) in N_2- and air-saturated PBS. AC1106-bound Ru(dmbpy)$_3^{2+}$ yielded similar results (see supporting information). As determined by the residuals shown above each plot, the luminescence decay traces of these species gave a satisfactory fit (- - -) to a single-exponential decay process assigned to the fully bound metal complex. Excited-state lifetimes for these species are given in Table 1.

Shown in Table 1 are the excited-state lifetimes of the free and AC1106-bound luminescent ruthenium(II) metal complexes in air- and N_2-saturated buffer (PBS: 10 mM phosphate, 120 mM NaCl, pH 7.4). Significantly, for both Ru(dmbpy)$(bpy)_2^{2+}$ and Ru(dmbpy)$_3^{2+}$, there is excellent agreement in the magnitudes of the amplification of the steady-state emission yield and in the enhancement in the excited-state lifetimes seen upon binding to AC1106. This agreement indicates that, in each case, the entire racemic sample of Ru(dmbpy)$(bpy)_2^{2+}$ and Ru-(dmbpy)$_3^{2+}$ is bound by the antibody. Thus, in the case that only a single enantiomer was bound the observed amplification in luminescence intensity would demand that the luminescence decay profiles were biphasic with one component having the lifetime of unbound metal complex and the other having a lifetime twice that found here for bound metal complex. On this basis, we can discount selective enantiomeric binding with this antibody and, instead, it appears that AC1106 binds both enantiomers in a nonstereospecific manner.[32] This result is surprising considering the high degree of stereospecificity that is often associated with antibody binding.[33]

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Table 1. Excited State Lifetimes of the Indicated Ruthenium(II) Metal Complex

metal complex	τ_a (ns)[a]	τ_b (ns)[b]	τ_c (ns)[c]	τ_d (ns)[d]	$\Delta_{s.s.}/\Delta_{t.r.}$[e]	K_a (M^{-1})
$Ru(dmbpy)(bpy)_2^{2+}$	310	460	690 [2.2]	840 [1.8]	2.1/2.2	1.2×10^7
$Ru(dmbpy)_3^{2+}$	210	320	620 [3.0]	670 [2.1]	2.6/3.0	$\geq 5 \times 10^7$
		640 (D_2O)		900 (D_2O)		
$Ru(bpy)_3^{2+}$	380	590	920 [2.4] (30%)[f]	1200 [2.0] (30%)[f]	n.d.[g]/2.4	7.7×10^3
$Ru(dmphen)_3^{2+}$	520	1800	2400 [4.6] (50%)[f]	4100 [2.3] (50%)[f]	n.d.[g]/4.6	8.4×10^5

[a] In air-saturated buffer (PBS). [b] In N_2-saturated PBS. [c] Bound by AC1106 in air-saturated PBS [enhancement of excited-state lifetime upon binding versus *a*]. [d] Bound by AC1106 in N_2-saturated PBS [enhancement of excited-state lifetime upon binding versus *b*]. [e] Amplification of integrated steady-state emission yield of the AC1106-bound to free metal complex measured in air-saturated PBS/enhancement of the excited-state lifetime of the AC1106-bound to free metal complex measured in air-saturated PBS. [f] Because of the lower affinity of AC1106 for this metal complex, these excited-state lifetime values were derived at the indicated partial saturation. [g] n.d. = not determined.

Single-exponential decay profiles were also maintained in the presence of oxygen, a known quencher of the excited state of $Ru(bpy)_3^{2+}$ and its simple derivatives.[19] In the event that each antibody-bound enantiomer were covered by AC1106 to different degrees, it seems reasonable to expect that these would show disparate accessibility toward O_2. In turn, this situation would give rise to non-exponential decay profiles.[34] We conclude, therefore, that AC1106 provides a similar degree of protection for the enantiomers of each metal complex.

Because of the high affinity of AC1106 for $Ru(dmbpy)_3^{2+}$ and the symmetrical nature of this metal complex, $Ru(dmbpy)_3^{2+}$ was chosen to probe the extent that the antibody-bound metal complex is protected from excited-state deactivation by solvent. Using a method developed previously to measure solvent accessibility of luminescent ruthenium(II) metal complexes in organized media,[35] the excited-state lifetimes of free and antibody-bound $Ru(dmbpy)_3^{2+}$ were measured in both N_2-saturated H_2O-PBS and D_2O-PBS (see Table 1). Using these values, it was determined that some 72% of the antibody-bound $Ru(dmbpy)_3^{2+}$ is inaccessible to excited-state deactivation *via* interaction with solvent. The protection from solvent extended to this metal complex by AC1106 is, in part, likely the result of the tight Van der Waals contact between the bound $Ru(dmbpy)_3^{2+}$ and protein contacts present in the antibody binding pocket. This characteristic property of antibody binding is known to exclude solvent from the region of contact between an antibody and its antigen.[36]

At the higher concentrations required for the time-resolved luminescence studies, a longer-lived component appeared when $Ru(bpy)_3^{2+}$ was titrated with a large excess of AC1106. As before, this component was assigned to the AC1106-bound metal complex. Deconvolution of the resulting biphasic luminescence decay trace yielded excited-state lifetimes for the antibody-bound $Ru(bpy)_3^{2+}$ in air- and N_2-saturated solution of 920 and 1200 ns, respectively. Because the concentration of both free and antibody-bound $Ru(bpy)_3^{2+}$ can be determined using the initial luminescent intensities of the two species, use of the time-resolved data allowed for a determination of the K_a for AC1106 binding to $Ru(bpy)_3^{2+}$ of $(7.7 \pm 2.4) \times 10^3$ M^{-1}.

Table 2. Association Constants (Listed in Descending Order) Derived *via* Competition ELISA for AC1106 and the Indicated Compounds[37]

compd	K_a (M^{-1})
$[Ru(mv^{2+}\text{-bpy})(bpy)_2]^{4+}$	2×10^8
1	8×10^6
$Ru(dmbpy)_3^{2+}$	3×10^6
5	2×10^5
mv^{2+}-bpy	8×10^4
$Ru(dmbpy)(bpy)_2^{2+}$	6×10^4

Similarly, when $Ru(dmphen)_3^{2+}$ was titrated with a large excess of AC1106, a long-lived component assigned to the antibody-bound metal complex was observed. Interestingly, the increase in the excited-state lifetime of the AC1106-bound $Ru(dmphen)_3^{2+}$ versus free $Ru(dmphen)_3^{2+}$ was significantly higher than that observed with the 2,2′-bipyridine derived metal complexes (see Table 1). When $Ru(phen)_3^{2+}$ was titrated with AC1106, no new component appeared. Unlike the methyl-free $Ru(bpy)_3^{2+}$ which bound to AC1106 weakly, no evidence for binding of $Ru(phen)_3^{2+}$ to AC1106 in a similar concentration regime was detected. Consequently, an upper limit to the association constant for AC1106 binding to $Ru(phen)_3^{2+}$ was set at 1×10^3 M^{-1}.

Competition ELISA Studies. While the luminescent ruthenium(II) metal complexes discussed above proved to be valuable probes of the molecular recognition properties of AC1106, steady-state and time-resolved luminescence methodology was not applicable to non-emissive compounds, such as $[Ru(mv^{2+}\text{-bpy})(bpy)_2]^{4+}$ or the hapten **1**. As a result, a competition enzyme-linked immunoabsorbant assay (ELISA) was used. This colorimetric assay monitors the quantity of antibody bound to a **1**-OVA conjugate on the surface of a microplate. By competing with binding sites on the plate, both luminescent and nonluminescent molecules can be used to inhibit the surface binding of AC1106. The resulting inhibition can then be quantified to yield the relative affinities that AC1106 has for a set of molecules. Hence, the assay signal is dependent on the amount of microplate-bound antibody, not on the intrinsic spectroscopic properties of a molecule of interest.

Table 2 shows the association constants for AC1106 binding to selected compounds as determined by competition ELISA. By comparing the affinities of AC1106 for various compounds, information on what features are important for recognition by AC1106 can be obtained. For example, as determined in the steady-state luminescence method, $Ru(dmbpy)_3^{2+}$ has a higher affinity than $Ru(dmbpy)(bpy)_2^{2+}$ for AC1106, again indicating the importance of methyl groups present on the metal ligands for complexation.[37] Furthermore, AC1106 has a higher affinity for $[Ru(mv^{2+}\text{-bpy})(bpy)_2]^{4+}$ than the pentacationic hapten **1**, a clear indication that more than simple charge−charge interactions are part of the binding process. In addition, AC1106

(32) The observed lack of stereospecific binding does not necessarily mean that the association constants for the two enantiomers are identical. However, it does imply that the binding pocket is sufficiently lacking in shape selectivity such that both enantiomers are accommodated.

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exhibits significantly higher affinities for both [Ru(mv^{2+}-bpy)-$(bpy)_2]^{4+}$ and the hapten **1** than the methyl viologen containing ligands, mv^{2+}-bpy and **5**, an indication that the presence of a metal center is a critical recognition element for AC1106. Finally, there is a dramatic preference of AC1106 for [Ru(mv^{2+}-bpy)$(bpy)_2]^{4+}$ over Ru(dmbpy)$(bpy)_2^{2+}$. When analyzed as the sum of its parts, [Ru(mv^{2+}-bpy)$(bpy)_2]^{4+}$ is equivalent to a Ru-(dmbpy)$(bpy)_2^{2+}$ metal complex to which methyl viologen has been synthetically "appended". Thus, the significant increase in the association constant between AC1106 and [Ru(mv^{2+}-bpy)$(bpy)_2]^{4+}$ indicates that AC1106 does indeed reserve part of its binding site for methyl viologen.

Quenching with Methyl Viologen and Oxygen. Because of the apparent affinity of AC1106 for methyl viologen, quenching experiments were designed to search for evidence of simultaneous binding of Ru(dmbpy)$(bpy)_2^{2+}$ and methyl viologen inside the binding pocket of AC1106. These experiments relied upon the fact that methyl viologen is an avid quencher of luminescence from ruthenium(II) metal complexes, due to light-induced electron transfer. Presumably, the close proximity of reactants bound within the same pocket would result in immediate (i.e. static) quenching of the triplet excited state of the metal complex (in this case, immediate refers to the time window within the 10-ns laser pulse).[38] In contrast, surface-bound methyl viologen could quench the triplet state of the metal complex located within the antibody pocket by way of a diffusional process. In the latter case, the triplet lifetime would decrease systematically with increasing concentration of viologen whereas in the former case the lifetime of the quenched species would be unresolvable from the excitation pulse.

When this experiment was performed, static quenching by methyl viologen was not observed. Instead, the AC1106-bound Ru(dmbpy)$(bpy)_2^{2+}$ was found to be subject to dynamic quenching (see Figure 6). However, in addition to the component assigned to the antibody-bound Ru(dmbpy)$(bpy)_2^{2+}$, a new component appeared with the addition of increasing amounts (10^{-3} M) of methyl viologen (see supporting information). Throughout the titration with methyl viologen, this new component was found to have a shorter excited-state lifetime relative to the AC1106-bound Ru(dmbpy)$(bpy)_2^{2+}$.[39] This new component was also found to be subject to dynamic quenching effects by methyl viologen. Furthermore, during the titration the concentration of this new species was observed to increase relative to that of the antibody-bound Ru(dmbpy)$(bpy)_2^{2+}$ (Figure 7).

A Stern−Volmer analysis proved useful in assigning this new species. Shown in Figure 6 is the Stern−Volmer plot of the excited-state lifetimes of the two components as a function of methyl viologen concentration. Bimolecular quenching constants (k_q's) for quenching by methyl viologen of 1.7×10^9 and 3.4×10^8 M^{-1} s^{-1} were measured for the new species and

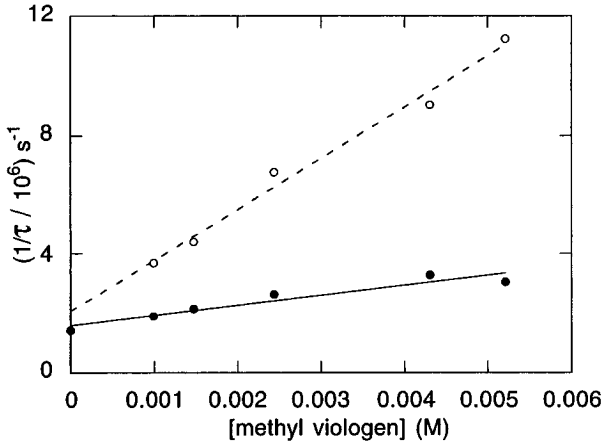

Figure 6. Stern−Volmer plot derived from the titration of the AC1106-Ru(dmbpy)$(bpy)_2^{2+}$ complex (1.4×10^{-5} M) with methyl viologen. Excited-state lifetimes of the antibody-bound (●) and free (○) Ru-(dmbpy)$(bpy)_2^{2+}$ were obtained from deconvolution of the biphasic luminescent decay traces observed in the presence of methyl viologen. Bimolecular quenching constants of 3.4×10^8 and 1.7×10^9 M^{-1} s^{-1} were measured for antibody-bound and free Ru(dmbpy)$(bpy)_2^{2+}$, respectively.

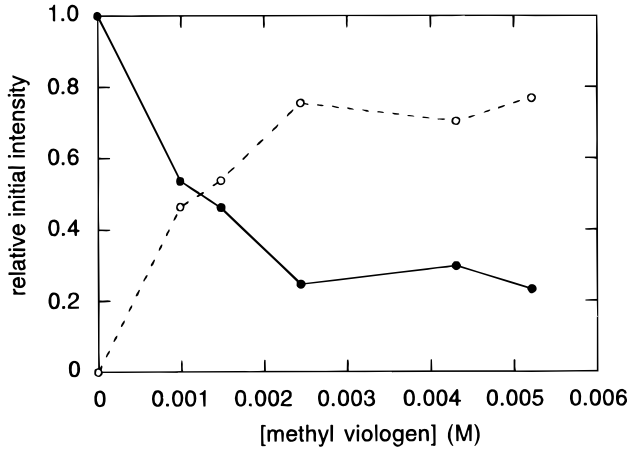

Figure 7. Plot of relative initial intensities of the AC1106−Ru(dmbpy)-$(bpy)_2^{2+}$ complex (●) and free Ru(dmbpy)$(bpy)_2^{2+}$ (○) as a function of the concentration of added methyl viologen. The concentration of each species is proportional to its respective initial luminescence intensity.

antibody-bound Ru(dmbpy)$(bpy)_2^{2+}$, respectively. The k_q for the new species was found to be identical, within experimental error, to the corresponding k_q for Ru(dmbpy)$(bpy)_2^{2+}$ measured in the absence of antibody (1.2×10^9 M^{-1} s^{-1}). Consequently, the new species is assigned as free Ru(dmbpy)$(bpy)_2^{2+}$.

Both the departure of the antibody-bound Ru(dmbpy)$(bpy)_2^{2+}$ in the presence of methyl viologen and the dynamic nature of the Stern−Volmer plot for the components shown in Figure 6 argue against the formation of a static, antibody-bound complex of Ru(dmbpy)$(bpy)_2^{2+}$ and methyl viologen. Interestingly, this displacement behavior is not unique to this antibody-based system.[40] Methyl viologen has also been shown capable of displacing polysulfonate-bound $Ru(bpy)_3^{2+}$.[41]

Quenching by O_2 was also investigated using $Ru(dmbpy)_3^{2+}$. Bimolecular quenching constants for quenching by O_2 were measured as 4.0×10^9 and 4.0×10^8 M^{-1} s^{-1} for the free and antibody-bound $Ru(dmbpy)_3^{2+}$, respectively. This reduction in the k_q for the antibody-bound $Ru(dmbpy)_3^{2+}$ can be easily accounted for by a decrease in the diffusion coefficient for the antibody-bound complex. In addition, the ability of AC1106 to shield the bound metal complex from oxygen, as it does with

(37) No significant, quantifiable inhibition was detected using $Ru(bpy)_3^{2+}$, $Ru(dmphen)_3^{2+}$, or methyl viologen despite solution evidence that they bind to AC1106. Competition ELISA is known to underestimate solution binding affinities. However, the relative order of affinities determined within the same assay is reliable. For a discussion of the caveats in using solid phase immunoassays to measure association constants, see: Butler, J. E. In *The Structure of Antigens*; Regenmortel, M. H. V., Ed.; CRC Press: Boca Raton, FL, 1992; Vol. I, pp 209−259.

(38) As a model for such an event, [Ru(mv^{2+}-bpy)$(bpy)_2]^{4+}$ in the presence or abscence of AC1106 was found to be nonluminescent on the nanosecond time scale. This indicates that the triplet excited state lifetime in either case is less than 10 ns. For more on the photophysics of [Ru-(mv^{2+}-bpy)$(bpy)_2]^{4+}$ see ref 60.

(39) Similar displacement behavior was seen with our previously reported polyclonal system[27] derived from **2** (unpublished results). Thus, it may be that this behavior is a general trend associated with this hapten design.

solvent, is also likely to play a role. A similar reduction in the k_q for quenching by O_2 has also been observed for DNA-bound versus free $Ru(phen)_3^{2+}$.[43]

Comparisons to Other Types of Organized Media. Enhanced luminescence has been seen with derivatives of $Ru(bpy)_3^{2+}$ and $Ru(phen)_3^{2+}$ when bound to certain hydrophobic, anionic media. For example, $Ru(phen)_3^{2+}$ has been proposed to bury one of its three 1,10-phenanthroline ligands into hydrophobic regions on the negatively-charged, double-stranded DNA scaffold.[42] As a consequence of this interaction, the luminescent lifetime of $Ru(phen)_3^{2+}$ increases in the presence of calf-thymus DNA from 470 to 1300 ns in air-saturated aqueous, buffered solution.[43] Similarly, the excited-state lifetime of $Ru(dmphen)_3^{2+}$ increases from 1700 ns in N_2-saturated H_2O to 3100 ns with partial immersion in a sodium dodecyl sulfate (SDS) micelle. This same trend in the presence of SDS is also observed for a variety of other 2,2′-bipyridine and 1,10-phenanthroline derived ruthenium(II) metal complexes.[35] Based on the complementary nature of antibody binding[44] and the hydrophobic, cationic nature of the hapten, one might predict the binding pocket of AC1106 to be both hydrophobic and anionic. As with DNA and with SDS, the change of the microenvironment inside the antibody binding pocket of AC1106 relative to the aqueous solution likely plays an important role in modulating the excited state lifetime of these metal complexes.

Other factors may also be responsible for the enhanced luminescence. The H_2O/D_2O experiment conducted with $Ru(dmbpy)_3^{2+}$ is clear evidence of the ability of AC1106 to protect the excited state of the antibody-bound metal complexes from deactivation *via* interaction with solvent. Along this same line of reasoning, AC1106 also seems to have the ability to protect the antibody-bound metal complex from excited-state deactivation by O_2. Finally, protein contacts with the bound metal complexes could result in a reduction of internal vibrational modes. In turn, the more rigidified metal complex would be less susceptible to excited-state deactivation caused by, for example, metal-to-ligand stretching modes.[45] In general, similar factors have been invoked to explain the enhanced emission seen when $Ru(phen)_3^{2+}$ binds to double-stranded DNA.[22b,46]

Interestingly, in our previously reported polyclonal antibody system elicited *via* immunization with **2**, the ruthenium(II) analogue of the cobalt(III)-based hapten used in this study, we measured excited-state lifetimes of 1300 and 1500 ns for the polyclonal antibody-bound $Ru(bpy)_3^{2+}$ in air- and N_2-saturated solution, respectively. The corresponding excited-state lifetimes for the AC1106−$Ru(bpy)_3^{2+}$ complex, namely 920 and 1200 ns, are noticeably shorter. A comparison of these two systems clearly indicates the excited-state lifetime of an antibody-bound $Ru(bpy)_3^{2+}$ complex is influenced by the nature of the antibody binding pocket.

AC1106 falls under the general category of metalloantibodies. In order to both modify and study the binding properties of antibodies, there has been an effort to incorporate metal cofactors into the binding sites of antibodies. Antibody binding pockets have been engineered to bind a variety of metals using site-directed mutagenesis.[47] In addition, antibodies elicited *via* immunization with metal-based haptens that bind metalloporphyrins,[48] metal chelates of ethylenediaminetetraacetic acid,[49] metal chelates of triethylenetetraamine,[28] and mercuric ions[50] have been reported. In many cases, seemingly subtle changes in the metal or metal chelate result in significant changes in the recognition properties of these antibodies.

AC1106 is not an exception to this observation. The results in this study indicate that AC1106 exhibits a very structure-dependent mode of binding. To some degree, this binding mode may be a consequence of the screening process used to find this monoclonal antibody. AC1106 was chosen by screening hybridoma cells derived from the immunization of the hapten **1** against **2**. Accordingly, the cross-reactivity of AC1106 is a compromise between one possible optimized fit for **1** and an accommodating fit for **2**. Indeed, the lack of stereospecific binding seen for $Ru(dmbpy)_3^{2+}$ and $Ru(dmbpy)(bpy)_2^{2+}$ may be a testament to the conciliatory position imposed by the screening process. It is possible that a highly specific immunological strategy to bind **1** does not conform to stereospecific binding of a seemingly analogous ruthenium(II) metal complex.

Considering the relatively large area that has been reported to be available to antibodies for surface−surface interactions between antibody and antigen (up to 880 Å^2)[51] and the potential for a variety of complementary interactions between **1** and AC1106, a large degree of the specificity of AC1106 is dependent on the presence or abscence of a surprisingly small hydrophobic portion (i.e. the methyl groups) of the luminescent metal complexes used in this study. This result is particularly relevant to the field of catalytic antibodies. Here, despite the relatively small size of haptenic transition state analogues, it has been observed that in some cases a small percentage of hapten-specific monoclonal antibodies are catalytic.[52] This observation, taken with the highly focused specificity of

(40) One possible explanation for the antibody-based displacement behavior is that disfavored electrostatic interactions result from simultaneously binding two dications next to each other in an antibody pocket. A second plausible explanation is a binding scheme in which the positively charged pyridine ring of methyl viologen affords some affinity for an antibody binding site that recognizes $Ru(dmbpy)(bpy)_2^{2+}$. In either case, it is possible that a dual hapten design that incorporates a neutral electron donor or acceptor tethered to $Ru(bpy)_3^{2+}$ may provide an antibody binding pocket capable of simultaneously binding the corresponding redox-active pair .

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AC1106, would seem to indicate that small regions in the structure of a ligand (or substrate) can dominate the antibody−antigen interactions. If these dominant interactions do not distinguish starting material from transition state, catalytic activity will be limited.[53]

Summary and Conclusion

The characterization of AC1106 elicited *via* immunization with a Co(dmbpy)(bpy)$_2^{3+}$−methyl viologen hapten (**1**) has been described. AC1106 was found cross-reactive for a variety of luminescent ruthenium(II) metal complexes which, when bound by this monoclonal antibody, exhibit a 2.2−4.6-fold enhancement in their excited-state lifetimes in air-saturated solution. AC1106 was found to be highly specific for methylated derivatives of Ru(bpy)$_3^{2+}$ and Ru(phen)$_3^{2+}$ in the order of Ru(dmbpy)$_3^{2+}$ > Ru(dmbpy)(bpy)$_2^{2+}$ > Ru(dmphen)$_3^{2+}$ > Ru(bpy)$_3^{2+}$ ≫ Ru(phen)$_3^{2+}$. The specificity of this antibody for these metal complexes is the result of a combination of factors that includes hydrophobicity and shape-selectivity. Through a combination of steady-state and time-resolved luminescence techniques, the affinities of AC1106 for these metal complexes were found to range over four orders of magnitude from $\geq 5 \times 10^7$ to $\leq 1 \times 10^3$ M^{-1}.

As demonstrated in this study, luminescent ruthenium(II) metal complexes, as model antibody ligands, are useful spectroscopic probes of antibody−antigen interactions. In particular, this study has focused on the unique recognition properties of a single monoclonal antibody. Other antibodies may exhibit considerably different recognition properties that result in stereospecific binding or even greater modulation of the excited-state lifetimes of Ru(bpy)$_3^{2+}$ and its derivatives. Based on the examples to date, the high specificity and affinity exhibited by this class of antibodies should be useful in the design of antibody-based systems that take advantage of the excited-state properties of Ru(bpy)$_3^{2+}$.

Experimental Section

Methods. (a) Luminescence Spectroscopy. All luminescence measurements were conducted in phosphate buffered saline (PBS: 10 mM sodium phosphate, 120 mM NaCl, pH 7.4). Steady-state luminescence spectra were recorded with a fully-corrected Perkin Elmer LS5 spectrofluorimeter. Solutions for steady-state luminescence studies were adjusted to possess an absorbance of less than 0.05 at the excitation wavelength of 450 nm and were air-equilibrated. Luminescence spectra were recorded between 500 and 800 nm and luminescence yields were determined by integration of the spectrum over the entire range. Where appropriate, Ru(bpy)$_3^{2+}$ was used as a reference compound.

All flash photolysis studies were made with a frequency-doubled, Q-switched Quantel YG481 Nd:YAG laser (pulse width 10 ns; maximum pulse energy 40 mJ). The laser intensity was attenuated with crossed-polarizers and all studies were carried out under conditions where the luminescence intensity was a linear function of laser power. Solutions were adjusted to possess an absorbance of *ca.* 0.05 at 532 nm and were purged continuously during the experiment with N_2, O_2, or mixtures thereof according to the needs of the experiment. Kinetic studies were carried out at a fixed wavelength of 610 nm with 20 individual laser shots being averaged, corrected for changes in the baseline, and analyzed with a microcomputer using nonlinear, least-squares iterative procedures. Reported kinetic parameters were theresult of a satisfactory fit to a single-exponential decay process, unless indicated otherwise, and were reproducible to within 5%. Biphasic decay profiles were analyzed according to $I_f(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$ where τ_1 and τ_2 refer to luminescence lifetimes of the two components and A_1 and A_2 refer to the initial luminescence intensities of the respective components.

(b) Competition ELISA. The hapten or inhibitor of interest was serially diluted by a factor of 2 in PBS across the length of a 8 × 12 well polypropylene microplate (Fisher Scientific) leaving one well near the center of each row free of hapten or inhibitor as a control. To each well was placed an aliquot (50 μL) of a 5×10^{-8} M solution of AC1106 in PBS. These solutions were allowed to incubate overnight at room temperature. At the end of this incubation period, an ELISA plate was blocked with 5 mg/mL of bovine serum albumin (BSA, Sigma) in PBS for 2 h at 37 °C and washed three times with PBS and shaken dry. The solutions were transferred row by row using a multichannel pipetter (1 × 12) to this plate and the incubation was continued for an additional 20 min. The plate was then washed three times with PBS and shaken dry. A solution of goat anti-mouse IgG (0.8 mg/mL) (H+L, Pierce Chemical Co.) was diluted 1:500 in a 5 mg/mL solution of BSA in PBS. Fifty microliters of this secondary antibody solution was portioned to each well and incubated for 1 h at 37 °C. The plate was then washed vigorously 10 times with PBS and shaken dry. Fifty microliters of 1-Step ABTS (2,2′-azinobis(3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt, Pierce Chemical Co.) was portioned to each well and was allowed to develop for approximately 10 min. The color change in each well was monitored spectrophotometrically at 405 nm using a EL311s automated microplate reader (BIO-TEK Instruments, Inc.).

(c) Association Constants. The equation to solve for the association constants using the steady-state luminescence data was derived from the simple equilibrium expressed in eq 1 where K_a is the association constant, X is the concentration of the AC1106-bound metal complex, R_f is the fixed concentration of metal complex (1×10^{-6} M), and C is the concentration of AC1106 in antibody binding sites. Because the change in emission intensity of the ruthenium(II) metal complex (E) observed upon titration with AC1106 is the product of the fraction of the total metal complex that is antibody-bound and the total difference in the integrated steady-state emission intensity of the antibody-bound and free metal complexes (E_c), eq 2 can be written. Solving for X in eq 1 and substituting into eq 2 yields eq 3. Using iterative, curve-fitting procedures (KaleidaGraph, version 3.0.2) and eq 3, association constants were derived from a plot of E versus C. After entering initial guesses for K_a and E_c, these variables were allowed to float freely over the iteration.

$$K_a = \frac{X}{(R_f - X)(C - X)} \tag{1}$$

$$E = E_c \frac{X}{R_f} \tag{2}$$

$$E = \frac{[(K_a C + K_a R_f + 1) - \sqrt{(K_a C + K_a R_f + 1)^2 - 4K_a^2 C R_f}]E_c}{2KR_f} \tag{3}$$

The equation to solve for the association constants using competition ELISA was derived from the relationships expressed in eqs 4 and 5. For eq 4 (analogous to eq 1), Y is the concentration of the AC1106−inhibitor complex, L is the concentration of the inhibitor, and C_f is the fixed concentration of AC1106 (1×10^{-7} M in antibody binding sites). The A_{405} value observed in the presence of inhibitor (A), proportional to the concentration of *free* antibody, is related to the fraction of bound antibody through eq 5 where A_o is the A_{405} value observed in the absence of inhibitor. Solving for Y in eq 4 and substituting into eq 5 yields eq 6. Using iterative, curve-fitting procedures and eq 6, association constants were derived from a plot of A versus L. After entering initial

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guesses for K_a and A_o, these variables were allowed to float freely over the iteration.

$$K_a = \frac{Y}{(L - Y)(C_f - Y)} \quad (4)$$

$$A = A_o - A_o\frac{Y}{C_f} \quad (5)$$

$$A = A_o - \frac{[(K_aL + K_aC_f + 1) - \sqrt{(K_aL + K_aC_f + 1)^2 - 4K_a^2LC_f}]A_o}{2K_aC_f} \quad (6)$$

Materials. (a) Preparation of Protein Conjugates. Hapten **1** (5 mg), 10 mg of keyhole limpet hemocyanin (Pierce Chemical Co.), and 50 μL of 0.1 M *N*-hydroxysulfosuccinimide (Pierce Chemical Co.) were placed in 0.9 mL total volume of doubly distilled water and adjusted to pH 7 with 0.1 M NaOH using a syringe. [1-Ethyl-3-(dimethylamino)propyl]carbodiimide (100 μL of 1 M) in double-distilled water was added and the solution was briefly vortexed. After 1 h at room temperature, 9.0 mL of PBS was added for a final protein concentration of 1 mg/mL and the resulting solution was dialyzed exhaustively against PBS at 4 °C using Spectra/Por 7 dialysis tubing, 50 000 MWCO (Spectrum). Ovalbumin (OVA) conjugates of **1** and **2** were prepared in a similar fashion.

(b) Production and Isolation of Antibodies. A male Balb/cJ mouse (Jackson Laboratories, Bar Harbor, ME) was injected intraperitonially with 150 μL of a 1 mg/mL solution of the hapten **1**−KLH conjugate in PBS emulsified with MPL + TDM adjuvant (RIBI Immunochem Research, Inc., Hamilton, MT). A total of four injections, 21 days apart, were performed. Fourteen days after the final injection, a final intravenous injection was performed with 200 μL of a 1 mg/mL solution of the hapten **1**−KLH conjugate in PBS 3 days prior to removal of the spleen. Hybridoma cells were produced from these spleen cells according to literature procedure[54] and were screened *via* ELISA[55] against both the **1**-OVA and **2**-OVA conjugates. One hybridoma line was found positive for both conjugates and was used in the production of ascites fluid. AC1106 was purified from ascites using NH_4SO_4 precipitation followed by protein G chromatography according to a previously published protocol.[56] Antibody concentration was determined spectroscopically using an A_{280} value of 1.4 for a 1.0 mg/mL rabbit antibody solution (ϵ_{280} = 220 000 M^{-1} cm^{-1}).[57]

(c) Preparation of ELISA Plates. In each well of a 8 × 12, polystyrene assay plate (Corning Glass Works, Corning, NY) 50 μL of a 0.5 μg/mL solution of the hapten **1**−OVA conjugate was allowed to evaporate at 37 °C overnight. Each well was fixed with 100 μL of MeOH for 30 min after which the MeOH was shaken out and the residual solvent was allowed to evaporate.

Syntheses. Proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a General Electric QE-300 NMR spectrometer using the residual peaks in the deuterated solvents as internal standards. Elemental analyses were performed by Atlantic Microlabs. Low-resolution FAB spectra were obtained using a Finnigan-MAT TSQ-70 instrument. 3-Nitrobenzyl alcohol was used as the matrix for FAB mass spectra. High-resolution mass spectra were recorded on a VG ZAB-2E instrument. Reverse-phase chromatography was performed with a FPLC system (Pharmacia LKB) using a HR10/10 column filled with PepRPC 15 μm (C_2/C_{18}).

Dichloromethane and acetonitrile were distilled from CaH_2 under N_2. All reagents were of the highest grade available and were purchased from the Aldrich Chemical Co. unless indicated otherwise. [Ru(4,4′-dimethyl-2,2′-bipyridine)$_3$]$^{2+}$ (Ru(dmbpy)$_3^{2+}$) and [Ru(4,7′-dimethyl-1,10′-phenanthroline)$_3$]$^{2+}$ (Ru(dmphen)$_3^{2+}$) were prepared according to the general procedure of Palmer and Piper for the synthesis of Ru(bpy)$_3^{2+}$.[58] [Ru(4,4′-dimethyl-2,2′-bipyridine)(2,2′-bipyridine)$_2$]$^{2+}$ (Ru(dmbpy)(bpy)$_2^{2+}$),[59] [Ru(mv^{2+}-bpy)(bpy)$_2$]$^{4+}$,[60] and mv^{2+}-bpy[60] (see Figure 1) were synthesized according to literature procedures. Ruthenium metal complexes were converted to their NO_3^- salts *via* purification on silica gel with 50:45:5 CH_3CN:H_2O:saturated KNO_3.[61] All literature compounds gave satisfactory ^{1}H NMR, ^{13}C NMR, and mass spectrometric analyses. The hapten **1** and its ruthenium(II) analogue **2** were synthesized according to the scheme shown in Figure 2.

[Co(2,2′-bipyridine)$_2$(1-(3-(4′-methyl-2,2′-bipyridin-4-yl)propyl)-1′-(carboxylpentyl)-4,4′-bipyridinium)]·(PF$_6^-$)$_5$ (1). Co(2,2′-bipyridine)$_2$Cl$_2$]Cl·H_2O[62] (19 mg, 0.038 mmol) and the metal ligand **5** (25 mg, 0.036 mmol) were dissolved in 3.0 mL of H_2O and allowed to stand at room temperature for 1.5 h whereupon the initially purple solution turned a light yellow-orange. Dioxane (0.7 mL) and 1.0 mL of trifluoroacetic acid were added and the resulting solution was allowed to stand for 40 min. The solvent was removed at room temperature under high vacuum and the resulting yellow oil was chromatographed using reverse-phase (C_2/C_{18}) chromatography. Two light yellow bands eluted using 15% CH_3CN/H_2O at a flow rate of 1.25 mL/min. The last yellow band to elute, according to ^{1}H NMR analysis, was the *tert*-butyl ester of **1**. The first yellow band to elute proved to be the hapten: ^{1}H NMR (CD_3OD) δ 1.48 (m, 2H, CH_2), 1.70 (m, 2H, CH_2), 2.12 (m, 2H, CH_2), 2.34 (t, 2H, COCH_2), 2.36 (m, 2H, bpy-CH$_2$CH_2), 2.70 (s, 3H, CH_3), 3.16 (t, 2H, bpy-CH_2), 4.78 (t, 2H, N$^+$-CH_2), 4.94 (t, 2H, N$^+$-CH_2), 7.31−9.40 (30H, m, aromatic *H*'s); ^{13}C NMR (CD_3OD) δ 21.5, 25.2, 26.5, 31.8, 32.1, 32.5, 34.4, 62.2, 63.0, 128.3, 128.4, 128.4, 128.6, 128.7, 129.4, 129.5, 132.6, 132.7, 133.3, 133.4, 145.0, 145.1, 147.1, 147.3, 151.2, 151.3, 151.5, 151.7, 152.5, 152.6, 156.5, 157.0, 157.3, 158.9, 159.0, 160.1, 160.2, 160.3, 177.0. For analytical purposes only, the hapten was converted to its PF_6^- salt and lyophilized to yield 17 mg (30%) of a light orange solid: Anal. Calcd for $C_{50}H_{50}CoF_{30}N_8O_2P_5$: C, 38.04; H, 3.19; N, 7.10. Found: C, 38.20; H, 3.56; N, 7.04.

[Ru(2,2′-bipyridine)$_2$(1-(3-(4′-methyl-2,2′-bipyridin-4-yl)propyl)-1′-(carboxylpentyl)-4,4′-bipyridinium)]·(PF$_6^-$)$_4$·4H$_2$O (2). The protected ruthenium(II) metal complex **6** (94 mg, 0.059 mmol) in 10 mL of 1:1 trifluoroacetic acid/water was allowed to stand at room temperature for 45 min. The solvent was removed using a rotary evaporator and the resulting solid purified on neutral alumina using a gradient of MeOH/H_2O. The purified solid was suspended in approximately 10 mL of H_2O, NH_4PF_6 was added, and the insoluble fraction was collected using vacuum filtration. The resulting orange solid was then taken up in dry CH_3CN and filtered to remove any insoluble inorganic salts. The solvent was removed using a rotary evaporator to yield 80 mg (88%) of the final product: ^{1}H NMR (CD_3CN) δ 1.40 (2*H*, m, CH_2), 1.62 (2H, m, CH_2), 2.03 (2H, m, CH_2), 2.28 (2H, t, COCH_2), 2.43 (2H, m, bpy-CH$_2$CH_2), 2.51 (3H, s, bpy-CH_3), 2.93 (2H, t, bpy-CH_2), 4.64 (2H, t, N$^+$-CH_2), 4.75 (2H, t, N$^+$-CH_2), 7.22−9.05 (30H, m, aromatic *H*'s); ^{13}C NMR (CD_3CN) δ 21.0, 24.5, 25.6, 31.2, 31.7, 39.9, 62.0, 62.5, 125.0, 125.9, 128.0, 128.1, 128.3, 129.1, 138.4, 146.4, 146.5, 150.6, 150.9, 151.3, 151.5, 152.0, 152.3, 152.4, 152.7, 157.1, 157.7, 157.8, 160.2, 160.7, 175.2; MS FAB, M$^+$: *m*/*z* 1331; HRMS, M$^+$: 1331.2004 (Calcd for $C_{50}H_{50}F_{18}N_8O_2P_3{}^{102}Ru$: 1331.2026). Anal. Calcd for $C_{50}H_{50}F_{24}N_8O_2P_4Ru·4H_2O$: C, 38.80; H, 3.78; N, 7.24. Found: C, 38.67; H, 3.60; N, 7.24.

***tert*-Butyl-6-bromohexanoate (3).** 6-Bromohexanoyl chloride (4.5 g, 21 mmol) and pyridine (1.8 mL, 22 mmol) were dissolved in 50 mL

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of CH_2Cl_2 and cooled to 4 °C. *tert*-Butyl alcohol (2.5 mL, 27 mmol) was added to the solution and stirred for 16 h at 4 °C whereupon a fine white precipitate of pyridine hydrochloride formed. The resulting suspension was washed with H_2O (3 × 100 mL), 10% K_2CO_3 (1 × 100 mL), and again with H_2O (1 × 100 mL). The organic layer was dried over Na_2SO_4 and distilled under high vacuum using a short path distillation apparatus to yield 4.1 g (78%) of a clear liquid: ^{1}H NMR δ ($CDCl_3$) 1.42 (s, 9H, C*H*$_3$), 1.44 (m, 2H, C*H*$_2$), 1.59 (m, 2H, C*H*$_2$), 1.83 (m, 2H, C*H*$_2$), 2.21 (t, 2H, C*H*$_2$CO), 3.39 (t, 2H, C*H*$_2$Br); ^{13}C NMR δ ($CDCl_3$) 24.1, 27.5, 28.0, 32.4, 33.5, 35.2, 80.1, 172.8; MS FAB, $[M + H]^+$: 251 *m*/*z*; HRMS, $[M + H]^+$: 251.0646 (calcd for $C_{10}H_{20}O_2Br$: 251.0647).

[1-(*tert*-Butoxycarbonylpentyl)-4,4′-bipyridinium]·(Br^-) (4). 4,4′-Bipyridine (1.1 g, 7.0 mmol) and *tert*-butyl 6-bromohexanoate (0.34 g, 1.4 mmol) were heated at reflux in 12 mL of dry CH_3CN for 48 h and the solvent was removed using a rotary evaporator. The resulting solid was purified on neutral alumina using a gradient of MeOH/$CHCl_3$ to give 0.2 mg (35%) of a tan solid: ^{1}H NMR δ ($CDCl_3$) 1.31 (s, 9H, C*H*$_3$), 1.37 (m, 2H, C*H*$_2$), 1.57 (m, 2H, C*H*$_2$), 2.06 (m, 2H, C*H*$_2$), 2.14 (t, 2H, C*H*$_2$CO), 4.97 (t, 2H, N^+-CH_2), 7.69 (d, 2H, $H_{3'}$ and $H_{5'}$), 8.41 (d, 2H, $H_{2'}$ and $H_{6'}$), 8.77 (d, 2H, H_3 and H_5), 9.72 (d, 2H, H_2 and H_6); ^{13}C NMR δ ($CDCl_3$) 24.0, 25.2, 27.9, 31.4, 34.8, 61.0, 80.1, 121.4, 125.8, 140.8, 145.9, 151.2, 172.6; MS FAB, $[M + H]^+$: 327 *m*/*z*; HRMS, $[M + H]^+$: 327.2072 (calcd for $C_{20}H_{27}N_2O_2$: 327.2073).

[1-(3-(4′-Methyl-2,2′-bipyridin-4-yl)propyl)-1′-(*tert*-butoxycarbonylpentyl)-4,4′-bipyridinium]·(Br^-)$_2$ (5). 4-(3-Bromopropyl)-4′-methyl-2,2′-bipyridine[60] (200 mg, 0.69 mmol) and [1-(*tert*-butoxycarbonylpentyl)-4,4′-bipyridinium]·(Br^-) (300 mg, 0.74 mmol) were heated at reflux in 25 mL of dry CH_3CN for 40 h whereupon a yellow precipitate formed. The suspension was cooled to room temperature and filtered using vacuum filtration. The precipitate was washed with diethyl ether (3 × 25 mL) and dried under vacuum to give 225 mg (45%) of a mustard yellow solid: ^{1}H NMR δ (CD_3OD) 1.42 (s, 9H, C(C*H*$_3$)$_3$), 1.45 (m, 2H, C*H*$_2$), 1.67 (m, 2H, C*H*$_2$), 2.10 (m, 2H, C*H*$_2$), 2.27 (t, 2H, C*H*$_2$CO), 2.45 (s, 3H, bpy-C*H*$_3$), 2.56 (m, 2H, C*H*$_2$), 2.97 (t, 2H, bpy-C*H*$_2$), 4.76 (t, 2H, N^+-CH_2), 4.88 (t, 2H, N^+-CH_2), 7.29 (1H, d, $H_{5'}$), 7.40 (1H, d, H_5), 8.09 (s, 1H, $H_{3'}$), 8.13 (s, 1H, H_3), 8.49 (d, 1H, $H_{6'}$), 8.54 (d, 1H, H_6), 8.63−8.67 (m, 4H, (viologen) H_3, $H_{3'}$, H_5, and $H_{5'}$), 9.27−9.34 (m, 4H, (viologen) H_2, $H_{2'}$, $H_{6'}$, and H_6), 9.32 (d, 2H,); ^{13}C NMR δ (CD_3OD) 21.2, 25.4, 26.6, 28.4, 32.2, 32.5, 32.8, 35.9, 62.7, 63.0, 81.5, 122.7, 123.7, 125.5, 126.3, 128.3, 147.1,147.2, 150.0, 150.5, 150.6, 151.2, 151.3, 152.3, 156.8, 157.3, 174.6; MS FAB, M^+: *m*/*z* 538; HRMS, M^+: 538.3295 (calcd for $C_{34}H_{42}N_4O_2$: 538.3308).

[Ru(2,2′-bipyridine)$_2$(1-(3-(4′-methyl-2,2′-bipyridin-4-yl)propyl)-1′-(*tert*-butoxycarbonylpentyl)-4,4′-bipyridinium)]·(PF_6^-)$_4$ (6). The metal ligand **5** (54 mg, 0.078 mmol) and *cis*-dichlorobis(2,2′-bipyridine)ruthenium(II) dihydrate (35 mg, 0.067 mmol) were heated at reflux in MeOH for 24 h and the solvent was removed using a rotary evaporator. The resulting reddish solid was taken up in 20 mL of H_2O and precipitated with NH_4PF_6. The precipitate was collected using vacuum filtration and purified on neutral alumina using a gradient of MeOH/CH_2Cl_2 to yield 81 mg (76%) of a bright orange solid: ^{1}H NMR δ (CD_3OD/CD_3CN) 1.38 (s, 9H, C(C*H*$_3$)$_3$), 1.41 (m, 2H, C*H*$_2$), 1.60 (m, 2H, C*H*$_2$), 2.03 (m, 2H, C*H*$_2$), 2.20 (t, 2H, C*H*$_2$CO), 2.45 (m, 2H, C*H*$_2$), 2.51 (s, 3H, bpy-CH_3), 2.94 (m, 2H, C*H*$_2$), 4.64 (t, 2H, N^+-CH_2), 4.78 (t, 2H, N^+-CH_2), 7.22−9.16 (30H, aromatic-H's); ^{13}C NMR δ (CD_3CN) 21.2, 25.0, 26.0, 28.3, 31.8, 32.0, 32.3, 35.7, 61.6, 62.3, 80.6, 125.2, 125.3, 125.4, 126.1, 126.9, 128.1, 128.4, 128.5, 128.7, 129.2, 138.6, 146.9, 147.4, 150.2, 150.4, 151.4, 151.6, 151.9, 152.5, 152.6, 152.9, 153.4, 157.7, 158.0, 173.5; MS FAB, M^+: 1242 *m*/*z*; HRMS, M^+: 1242.3021 (calcd for $C_{54}H_{58}F_{12}N_8O_2P_2{}^{102}Ru$: 1242.3010).

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Supporting Information Available: Addition spectra and data plots (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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