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Luminescent Ruthenium(II)− and Rhenium(I)−Diimine Wires Bind Nitric Oxide Synthase

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Abstract: Ru(II)− and Re(I)−diimine wires bind to the oxygenase domain of inducible nitric oxide synthase (iNOSoxy). In the ruthenium wires, $[Ru(L)_2L']^{2+}$, L′ is a perfluorinated biphenyl bridge connecting 4,4′-dimethylbipyridine to a bulky hydrophobic group (adamantane, **1**), a heme ligand (imidazole, **2**), or F (**3**). **2** binds in the active site of the murine iNOSoxy truncation mutants Δ65 and Δ114, as demonstrated by a shift in the heme Soret from 422 to 426 nm. **1** and **3** also bind Δ65 and Δ114, as evidenced by biphasic luminescence decay kinetics. However, the heme absorption spectrum is not altered in the presence of **1** or **3**, and Ru−wire binding is not affected by the presence of tetrahydrobiopterin or arginine. These data suggest that **1** and **3** may instead bind to the distal side of the enzyme at the hydrophobic surface patch thought to interact with the NOS reductase module. Complexes with properties similar to those of the Ru−diimine wires may provide an effective means of NOS inhibition by preventing electron transfer from the reductase module to the oxygenase domain. Rhenium−diimine wires, $[Re(CO)_3L_1L_1']^{+}$, where L_1 is 4,7-dimethylphenanthroline and L_1' is a perfluorinated biphenyl bridge connecting a rhenium-ligated imidazole to a distal imidazole (F_8bp-im) (**4**) or F (F_9bp) (**5**), also form complexes with Δ114. Binding of **4** shifts the Δ114 heme Soret to 426 nm, demonstrating that the terminal imidazole ligates the heme iron. Steady-state luminescence measurements establish that the **4**:Δ114 dissociation constant is 100 ± 80 nM. Re−wire **5** binds Δ114 with a K_d of 5 ± 2 μM, causing partial displacement of water from the heme iron. Our finding that both **4** and **5** bind in the NOS active site suggests novel designs for NOS inhibitors. Importantly, we have demonstrated the power of time-resolved FET measurements in the characterization of small molecule:protein interactions that otherwise would be difficult to observe.

Introduction

The enzyme nitric oxide synthase (NOS) is the major biological source of nitric oxide (NO), a secondary messenger acting in a myriad of circumstances that include neuronal development, regulation of blood pressure, apoptosis, neurotransmission, and immunological response.[1−7] Because of the central importance of NO, NOS has been implicated in septic shock, inflammation, a variety of neurodegenerative disorders, and heart disease.[8−10]

Full-length NOS consists of oxygenase, reductase, and calmodulin binding domains. The NOS oxygenase domain (NOSoxy), which contains cysteine-ligated heme and tetrahydrobiopterin (H_4B) cofactors, catalyzes the conversion of arginine and molecular oxygen to NO and citrulline.[11] The electrons necessary for this reaction are provided by the reductase module, which is attached to NOSoxy by a calmodulin-binding linker.[12,13] NOS functions as a homodimer; the reductase module from one half of the dimer reduces the oxygenase domain of the other.[14,15] Calmodulin binding is

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known to modulate electron transfer and, hence, catalysis.[16−18] Several crystal structures of the NOS oxygenase domain have been determined,[19−21] but the structure of the full-length enzyme remains elusive.

We have a long-standing interest in the high-valent intermediates thought to play key roles in heme-mediated oxidations.[22−29] To observe these intermediates, we have designed Ru−diimine photosensitizers (Ru−wires) that bind to the mechanistically related enzyme cytochrome P450 and inject an electron into the active site upon excitation with 470-nm light.[27] Energy transfer between the excited state of the Ru−wire and the heme also serves as a sensitive structural probe.[22,29] Like NOSoxy, cytochrome P450 enzymes possess a cysteine-ligated heme in the active site and catalyze the oxidation of substrates using molecular oxygen and two electrons supplied by a reductase (in the case of P450, a separate protein).[30]

Given the postulated mechanistic similarities between NOS and cytochrome P450, we have endeavored to develop similar photosensitizer−wires for NOS. Our initial investigation showed that complexes **1**−**3** bind the oxygenase domain of murine inducible NOS (iNOSoxy) with micromolar dissociation constants. Intriguingly, a combination of fluorescence energy transfer (FET) measurements and structural modeling suggests that **1** and **3** bind to the surface patch thought to interact with the reductase module. Second generation compounds **4** and **5**, which are structurally analogous to **2** and **3**, bind in the iNOSoxy active site with micro- and nanomolar dissociation constants.

Experimental Section

Murine inducible NOS oxygenase domain constructs with N-terminal truncations at residues 65 (Δ65) and 114 (Δ114) were prepared as previously described.[31] Small aliquots of the protein solutions were exchanged into phosphate buffer (50 mM potassium phosphate, 100 mM potassium chloride, pH 7.2) using a desalting column immediately before use. The measurement of the heme Soret peak at 422 nm verified successful removal of the dithiothreitol (DTT) present in the storage buffer.

High-spin, dimeric Δ65 iNOS was generated by incubating Δ65 with 1 mM tetrahydrobiopterin (H_4B) and 1 mM arginine (Arg) for 2 h at 4 °C before diluting the sample to final concentrations of 0.1 mM H_4B and 1 mM Arg. Satisfactory Arg and H_4B binding was signaled by a shift of the Soret to 396 nm. NOS extinction coefficients were determined using the hemochromogen assay; 1 mL of NOS solution was diluted with 0.125 mL of 0.5 M NaOH and 0.125 mL of pyridine, and then reduced with several grains of sodium dithionite. The resulting ferrohemochromogen concentration was calculated using an extinction coefficient of 31 mM^{-1} cm^{-1} at 556 nm. The assays were calibrated using cytochrome P450cam (ϵ_{416} = 115 mM^{-1} cm^{-1}).[32] The NOS extinction coefficients calculated using this method are substrate-free Δ65 (−H_4B, −Arg) ϵ_{422} = 75 mM^{-1} cm^{-1}, Δ65 (+H_4B, +Arg) ϵ_{396} = 75 mM^{-1} cm^{-1}, and substrate-free Δ114 ϵ_{422} = 85 mM^{-1} cm^{-1}.

Ru−wires (**1**−**3**) were prepared by the literature procedure.[27] The fluorinated biphenyl bridging moieties for Re−wires **4** and **5** were synthesized by reacting imidazole and perfluorobiphenyl in dimethyl sulfoxide. The resulting mono- and disubstituted perfluorobiphenyl−imidazole ligands were separated by flash silica chromatography. Re(dimethylphenanthroline)$(CO)_3$Cl was treated with silver triflate, and then reacted with either the mono- or disubstituted perfluorobiphenyl−imidazole ligand to form **4** and **5** as triflate salts.[33]

Both time-resolved and steady-state spectroscopic measurements were performed as previously described.[27] Luminescence decay profiles were fit to a biexponential function (eq 1):

$$I(t) = c_1\, e^{-k_1 t} + c_2\, e^{-k_2 t} \quad (1)$$

using a nonlinear least-squares algorithm. The ratio of enzyme-bound to free ruthenium complex is c_1:c_2, where k_1 and k_2 are the luminescence decay rate constants for the enzyme-bound and free ruthenium complexes. Dissociation constants were derived from c_1:c_2 as previously described.[27]

Characteristic FET distances (R_0) for the Ru− and Re−diimine wires with iNOSoxy were calculated from the probe emission and NOS absorption spectra.[27] These distances are 24.3 Å for **1** and **2** with Δ114, 19.6 Å for **3** with Δ114, 32 Å for **4** and **5** with Δ114, 24.3 Å for **1** and **2** with substrate-free Δ65, 19.5 Å for **3** with substrate-free Δ65, 23.9 Å for **1** and **2** with Arg- and H_4B-bound Δ65, and 19.3 Å for **3** with Arg- and H_4B-bound Δ65.

Results and Discussion

Ru−Wires. Two murine iNOSoxy truncation mutants, Δ114 and Δ65, were investigated. Importantly, Δ114 is predominantly monomeric, while Δ65 exists in a monomer−dimer equilibrium, forming a strong dimer in the presence of H_4B.[31] For clarity, both Δ65 without bound H_4B and Δ114 are referred to below as "monomeric". Monomeric iNOSoxy has an exposed active site (see below), while dimeric iNOSoxy has a much more constricted substrate access channel.

No change in the iNOSoxy heme absorption spectrum was observed upon stoichiometric addition of **1** or **3** to either Δ114 or Δ65. In contrast, the addition of excess **2** to Δ114 or monomeric Δ65 (−H_4B, −Arg) resulted in a heme Soret shift from 420 or 422 to 426 nm, consistent with imidazole ligation of the heme (Figure 1). The absorption spectrum of dimeric Δ65 (+H_4B, +Arg) was not altered in the presence of **1**−**3**, indicating that none of the Ru−wires displaces Arg from the dimeric iNOSoxy active site.

In all cases, biexponential Ru−wire luminescence decays were observed in the presence of stoichiometric Δ114 or Δ65,

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Table 1. Ru−Wire Dissociation Constants and Ru−Fe Distances from FET Kinetics[a]

	Δ114		Δ65		Δ65 +Arg, +H_4B	
compound	K_d (μM)	Ru–Fe (Å)	K_d (μM)	Ru–Fe (Å)	K_d (μM)	Ru–Fe (Å)
1	0.88 ± 0.15	18.9 ± 0.1	0.54 ± 0.04	19.6 ± 0.2	1.7 ± 0.4	19.6 ± 0.4
2	7.1 ± 0.4	17.8 ± 0.5	6.5 ± 2.4	19.3 ± 0.6	7.2 ± 3.4	20.9 ± 0.8
3	0.71 ± 0.09	20.1 ± 0.1	0.58 ± 0.16	20.2 ± 0.4	0.89 ± 0.15	21.0 ± 0.3

[a] Uncertainties are the root-mean-square deviations calculated from independent measurements (3 for Δ114, 2 for Δ65, 3 for Δ65 +H_4B, +Arg).

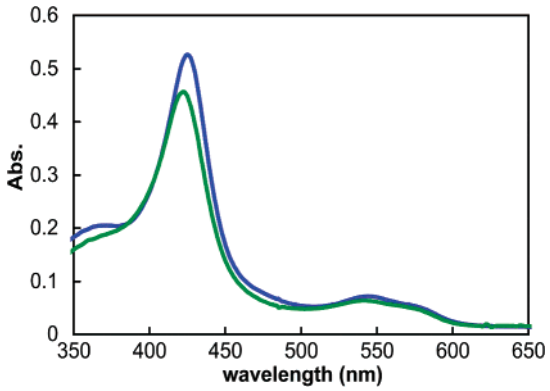

Figure 1. UV−visible absorption spectrum of Δ114 alone (5.7 μM; green) and bound to **2**, corrected for the absorption attributable to the Ru−wire (+20.5 μM **2**; blue).

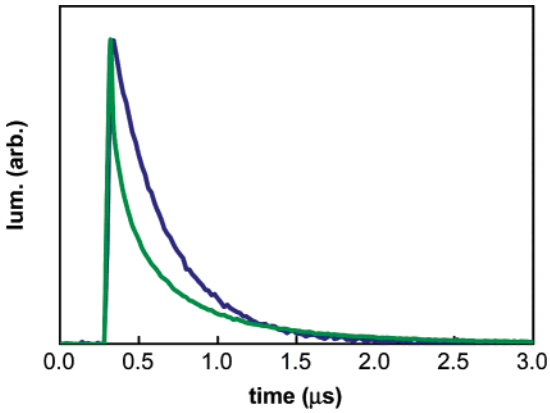

Figure 2. Sample transient luminescence decay data for **1** (blue) and a 1:1 mixture of **1** and Δ65 (1.8 μM; green). The fast component of the luminescence decay corresponds to **1** bound to Δ65.

indicating that the Ru−wires bind to the enzyme (Figure 2). Quenching of the Ru−wire excited state could, in principle, occur by either energy or electron transfer to the iNOSoxy heme. Since no electron-transfer products were observed by transient UV−visible absorption spectroscopy, and because the rate of luminescence quenching is consistent with FET, energy transfer is the probable mechanism of Ru−wire luminescence quenching. The weightings of the fast and slow luminescence decay phases were used to calculate dissociation constants, while the rates of energy transfer were used to calculate Ru to heme−Fe distances. Ru−Fe distances so obtained for Ru−wire:P450cam conjugates matched those observed in the corresponding crystal structures to within 0.4 Å.[22]

The Ru−wires bind with micromolar dissociation constants and with Ru−Fe distances of 18−21 Å (Table 1). Interestingly, **1** and **3** bind Δ114, Δ65, and dimeric Δ65 (+H_4B, +Arg) with dissociation constants that are virtually identical. The Ru−Fe distances calculated for **1** and **3** are similar for Δ114 and Δ65 and are unaffected by the presence of H_4B and Arg (Table 1).

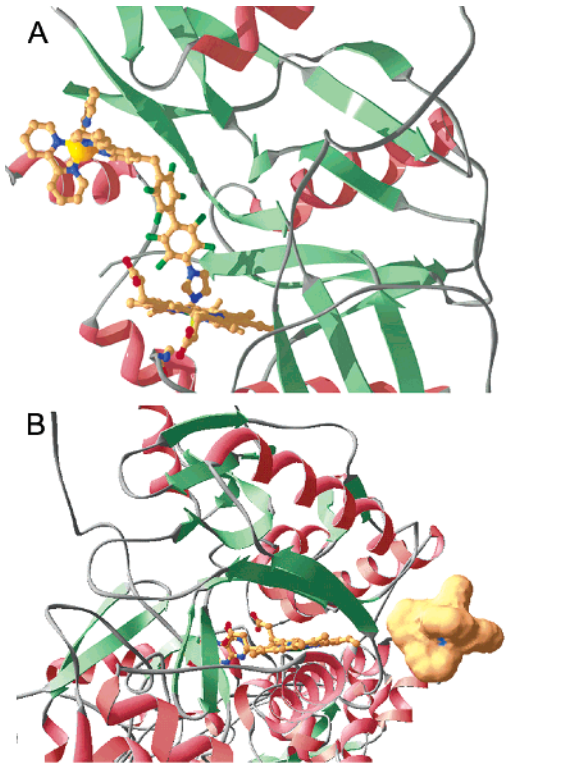

Figure 3. (A) Model of **2** bound to the exposed heme of monomeric Δ114. The Ru−Fe distance in this model is 16.9 Å. (B) The NOS dimer, shown with $Ru(bpy)_3$ (van der Waals surface) docked at the proposed reductase binding site. The Ru−Fe distance is ∼19 Å, consistent with the Ru−Fe distances measured for the Δ65:**1** and Δ65:**3** conjugates.

The spectroscopic evidence suggests that **1** and **3** do not bind in the active site; the heme absorption spectrum is not altered in the presence of **1** or **3**, and the K_d values and Ru−Fe distances determined for these Ru−wires are not substantially altered by the addition of H_4B and Arg. Addition of stoichiometric **4**, which binds in the iNOS active site (see below), does not alter the binding of **3** to monomeric Δ65, again demonstrating that **3** does not bind in the active site (Supporting Information).

Consistent with these data, modeling suggests that **1** and **3** cannot fit into the substrate access channel of dimeric Δ65 (+H_4B, +Arg), owing to the bulk of the ruthenium tris-bipyridyl moiety. Instead, the calculated Ru−Fe distances indicate that **1** and **3** may bind on the distal side of the enzyme, at the proposed binding site of the reductase module (Figure 3b).[14] The proposed binding site is concave and hydrophobic. The Ru−wires present few opportunities for specific interactions with the protein surface. Instead, extensive wire:protein hydrophobic contacts likely mediate binding. Of interest in this regard is that other Ru^{II}−diimines bind cytochrome *c* oxidase at the physiologically relevant cytochrome *c* binding site.[34,35]

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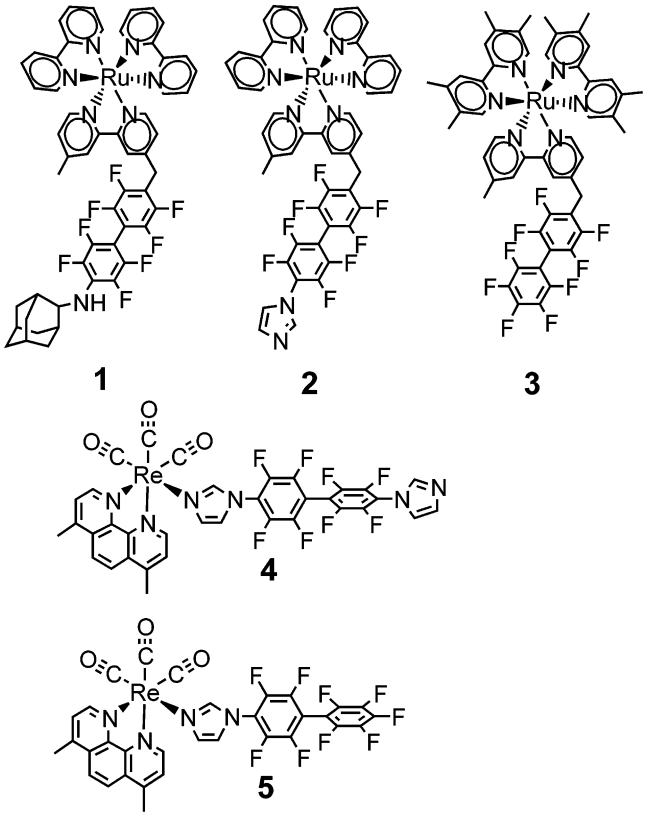
Chart 1. Ru−Wires (**1**−**3**) and Re−Wires (**4** and **5**) Bind iNOSoxy and also Cytochrome P450cam



In contrast, the shifts in the absorption spectra of monomeric Δ65 and Δ114 in the presence of excess **2** clearly indicate that the imidazole of **2** ligates the heme (Figures 1 and 3a).[19] Structural modeling suggests that the less bulky **2**, which lacks the adamantyl group of **1** and the tetramethylbipyridine ligands of **3**, can bind in the more exposed active site of monomeric iNOSoxy (Figure 3a). However, **2** may also bind iNOSoxy at the site occupied by **1** and **3**; 1.5 equivalents of **2** bind to monomeric Δ65 (−H_4B, −Arg) when the Ru−wire is present in 6-fold excess, and **2** binds to dimeric Δ65 (+H_4B, +Arg), in which the active site is occupied. In contrast to those in **1** and **3**, the Ru−Fe distances determined for the **2**:Δ114 and **2**:Δ65 +H_4B, +Arg conjugates increase markedly from 17.8 to 20.9 Å, suggesting a different mode of binding to monomeric (Δ114) and dimeric (Δ65 +H_4B, +Arg) iNOSoxy. These data together suggest that **2** binds to both the active site of monomeric iNOSoxy and another portion of the protein with similar K_d values.

Re−Wires. Luminescent rhenium diimines, $[Re(CO)_3(L_2)(L_2')]^+$, where L_2 is a 2,2′-bipyridyl or phenanthryl derivative, and L_2' is a nitrogen donor, such as imidazole or pyridine, typically have microsecond excited-state lifetimes. Like $Ru(bpy)_3^{2+}$, the excited states are both good oxidants (~1.2 V vs NHE) and reductants (−0.7 V vs NHE).[36] In addition, photochemically generated $[Re(CO)_3(L_2)(L_2')]^{2+}$ complexes are extremely strong oxidants (~1.8 V vs SCE).[37]

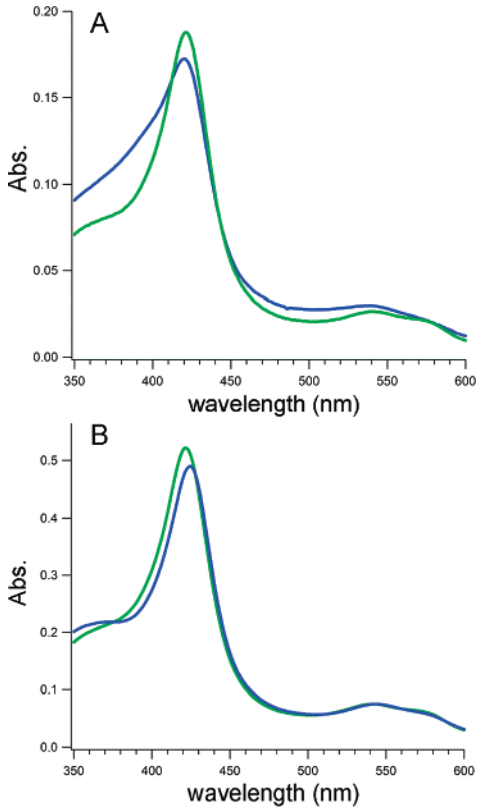


Figure 4. (A) 2.2 μM Δ114 alone (green) and with stoichiometric **5** (blue). Both the slight shift in peak wavelength and development of a pronounced shoulder indicate partial conversion to high-spin, five-coordinate heme. (B) 6.0 μM Δ114 alone (green) and with stoichiometric **4** (blue). The red shift in peak wavelength is consistent with imidazole ligation of the heme.

To take advantage of rhenium photochemistry, we synthesized Re−wires **4** and **5** (Chart 1). The complexes are structurally similar to **1** and **3**, but the Re−photosensitizer is smaller. The surface areas of $[Ru(bpy)_3]^{2+}$ and $[Re(CO)_3$(dimethylphenanthroline)(imidazole)$]^+$ are 650 and 550 Å^2, respectively.[38] Additionally, the *fac*-carbonyls of **4** and **5** result in a more compact profile on one side of the complex, allowing a closer approach to the protein. The virtually identical absorption spectra of **4** and **5** are typical of Re−diimines. Both complexes are luminescent, with emission spectra centered at 560 nm (quantum yields are 0.055 in phosphate buffer).

Upon addition of **4** to Δ114 murine iNOSoxy, the Soret shifts from 422 to 426 nm, signaling imidazole ligation to the heme iron (Figure 4b). Time-resolved luminescence measurements indicate that **4** is almost completely bound to NOS in 1:1 micromolar solutions (data not shown). Indeed, a K_d could not be determined from the luminescence decay data due to the rapidity of the decay[39] and the almost complete absence of a slow decay rate corresponding to free **4** in solution. Instead, a dissociation constant of 100 ± 80 nM was calculated from a comparison of the steady-state luminescence spectra of **4** alone and bound to Δ114 iNOSoxy (Figure 5).

Re−wire **5** causes a blue shift in the Δ114 Soret, indicating partial conversion to a high-spin, five-coordinate heme (Figure 4a). In addition to a small shift in peak wavelength, a noticeable shoulder appears in the Soret (Figure 4a), consistent with partial

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(39) Initial data suggest that the luminescence quenching is attributable to rapid electron transfer (ref 33).

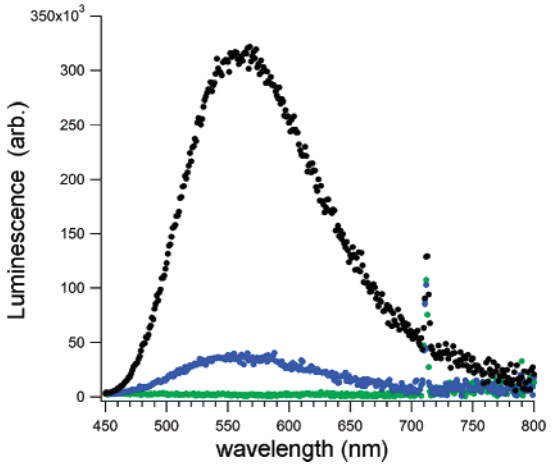

Figure 5. Steady-state luminescence spectra of 2.6 μM samples of Δ114 (green), **4** (black), and a 1:1 mixture of Δ114 and **4** (blue). The luminescence of **4** is strongly quenched in the presence of Δ114, making it a sensitive indicator of the presence of the enzyme. The sharp spike at 710 nm is an artifact arising from scattered 355-nm excitation light.

displacement of the iron-ligating water from the active site. The time-resolved luminescence decay spectra (Supporting Information) suggest that **5** binds with a dissociation constant of 5 ± 2 μM and a Re−heme distance of ~18 Å. Both the changes in the Soret absorption and the calculated Re−Fe distance are consistent with **5** binding in the active site. Structural modeling indicates that **5** can fit in the active site of monomeric iNOSoxy, unlike the sterically bulkier **3**. The structural dissimilarities of **5** and Arg make it surprising that **5** binds at all. However, the relatively exposed active site of the monomeric Δ114 iNOSoxy provides good surface complementarity with the fluorinated biphenyl moiety.

Concluding Remarks

Small molecules that interfere with NOS function are being investigated as potential treatments for several diseases.[40,41] All currently known inhibitors in this class bind in the active site of the enzyme. In contrast, Ru−wires or similar compounds may provide an effective means of NOS inhibition by preventing electron transfer between the reductase module and oxygenase domain. Inhibitors that disrupt protein:protein interactions could be of great value, owing to the biological ubiquity of transient protein complexes.

The interactions of **2** and **4** with Δ114 are in many ways analogous to those observed with small molecules that prevent NOS holoenzyme dimerization.[42] Because only iNOS exhibits an appreciable monomer−dimer equilibrium in vivo, these inhibitors are highly isoform selective.[42] The low dissociation constant of **4** makes it a useful lead compound for further development. The ~70-fold difference in dissociation constants between **2** and **4** illustrates the steric influence of the $Ru(bpy)_3^{2+}$ moiety.

The ability of **5** to bind in or near the iNOSoxy active site is remarkable given its dissimilarity to Arg or known inhibitors. As with the Ru−wires, it seems likely that binding is driven principally by hydrophobic interactions. Although **3** binds more tightly than **5** to NOS, it does not produce a similar shift in the absorption spectrum, again demonstrating the importance of steric bulk in modulating wire−NOS interactions.

Most notably, we have shown the usefulness of FET kinetics in characterizing small molecule:protein interactions. Conventional UV−visible absorption measurements or competition binding assays would have overlooked the ability of **1** and **3** to bind iNOSoxy. What is more, FET measurements yield distance constraints, which provide additional information about the interaction between the wire and target enzyme. As demonstrated by these results, FET data greatly facilitate the formulation of structural models for wire:enzyme conjugates.

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Supporting Information Available: Further transient luminescence decay kinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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