Controlling the Optical and Catalytic Properties of Artificial Metalloenzyme Photocatalysts Using Chemogenetic Engineering

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Abstract

Visible light photocatalysis enables a broad range of organic transformations that proceed via single electron or energy transfer. Metal polypyridyl complexes are among the most commonly employed visible light photocatalysts. The photophysical properties of these complexes have been extensively studied and can be tuned by modifying the substituents on the pyridine ligands. On the other hand, ligand modifications that enable substrate binding to control reaction selectivity remain rare. Given the exquisite control that enzymes exert over electron and energy transfer processes in nature, we envisioned that artificial metalloenzymes (ArMs) created by incorporating Ru(II) polypyridyl complexes into a suitable protein scaffold could provide a means to control photocatalyst properties. This study describes approaches to create covalent and non-covalent ArMs from a variety of Ru(II) polypyridyl cofactors and a prolyl oligopeptidase scaffold. A panel of ArMs with enhanced photophysical properties were engineered, and the nature of the scaffold/cofactor interactions in these systems was investigated. These ArMs provided higher yields and rates than Ru(Bpy)₃²⁺ for the reductive cyclization of dienones and the [2+2] photocycloaddition between C-cinnamoyl imidazole and 4-methoxystyrene, suggesting that protein scaffolds could provide a means to improve the efficiency of visible light photocatalysts.

Keywords

Visible-light photocatalysis, biocatalysis, artificial metalloenzyme, metal polypyridine complex

Introduction

Visible light photocatalysts enable a wide range of chemical reactions and provide access to compounds that are difficult to produce using other synthetic methods.^{1,2} After photoexcitation, these catalysts can react with substrates via single-electron transfer (SET) or energy transfer (ET) to produce high energy intermediates. In the former case, photoredox catalysis is enabled by a second SET event with a sacrificial oxidant or reductant (the order of reaction with the substrate and oxidant/reductant can also be reversed), while in the latter case, the bidirectional nature of ET leads to catalyst turnover. Both organic^{3,4} and inorganic⁵ molecules are used as photocatalysts, but metal polypyridyl complexes like Ru(Bpy)₃²⁺, Ir(Ppy)₃, and derivatives thereof are particularly valuable for synthetic organic methodology⁶. Upon absorbing visible light, these complexes efficiently form excited states that are suitably long-lived to enable reaction with organic substrates.^{7,8} Furthermore, metal polypyridyl complexes exhibit reversible redox properties and are not prone to decomposition or photobleaching.⁵

Structure-activity relationships have provided insight into the effects of polypyridine substituents on the photophysical and electronic properties of metal polypyridine complexes.^{5,9} Ligand modification has also been used to tune the selectivity of these complexes in reactions involving organic substrates.¹⁰ Given that many photocatalysts are only involved in the initial electron or

energy transfer to/from a substrate and that the resulting high energy intermediates can undergo rapid subsequent reactions, the substrate must either be in a chiral environment during the excitation step and remain there during the selectivity-determining step or later engage with a chiral co-catalyst during the selectivity-determining step.¹¹ Reactions proceeding via electron transfer can also proceed via radical chain mechanisms that do not involve the catalyst and compete with the desired closed catalytic cycle to erode catalyst controlled selectivity.^{12,13}

Many enzyme-catalyzed reactions,^{14,15} including recently reported examples of non-native photocatalytic reactions,¹⁶ are initiated via SET, and highly efficient ET occurs in photosystems I and II¹⁷. The remarkable selectivity and specificity of these systems results from their ability to bind intermediates and cofactors throughout their respective catalytic cycles. On the other hand, the functional properties of such systems are limited relative to the rapidly expanding repertoire of synthetic photoredox^{18,19} and energy transfer³ catalysts. A variety of approaches have therefore been taken to explore the photophysical and photocatalytic capabilities of metal polypyridine complexes in the context of protein scaffolds.²⁰ For example, Gray has extensively studied SET in Ru(II)-polypyridyl modified-P450 BM3 enzymes²¹ and other scaffolds^{22–24}. Cheruzel later developed related systems to enable light-driven P450 hydroxylation catalysis²⁵ and to modulate the photophysical and catalytic properties of these systems through pyridyl ligand substitution²⁶. Ward linked Ru(II)-polypyridyl complexes to (strept)avidin via non-covalent biotin binding²⁷ or covalent cysteine bioconjugation^{28,29} and explored SET in donor-photosensitizer-acceptor triads involving these systems. Importantly, in these cases, the metal polypyridine complexes were solvent exposed, often residing in shallow clefts with limited capacity to interact with the cofactor. While changes in the photophysical or redox properties of the protein-linked complexes were reported in these examples^{20–29} and others^{30–33}, none of these systems were used as photocatalysts for direct transformations of organic substrates.

We envisioned that site-specific incorporation of metal polypyridine complexes into a suitable protein scaffold might enable greater control over the photophysical and catalytic properties of these complexes. Herein, we report the synthesis and characterization of a series of artificial metalloenzymes (ArMs) comprising different Ru(Bpy)₃²⁺ cofactors linked either covalently (**1a-f**) or non-covalently (**3a-i**) to a prolyl oligopeptidase (POP)³⁴ from *Pyrococcus furiosus* (*Pfu*)³⁵ (Figure 1a). Systematic manipulation of both the cofactor and the POP scaffold were conducted to establish how structural changes impacted cofactor binding and photophysical properties. Improved binding, luminescence intensities, and luminescence lifetimes were observed for ArM variants due to hydrophobic and electrostatic interactions with residues in the POP active site. The catalytic activity of a panel of covalent ArMs was then evaluated using a reductive cyclization reaction proceeding via SET and a [2+2] cycloaddition enabled by ET. Yields of the desired products and reaction rates were increased by the engineered ArMs in both cases. These results indicate that POP variants can both modulate metal polypyridyl photophysical properties and improve photocatalysis yields, highlighting their potential as scaffolds for the further development of ArM photocatalysts.

Results

Scaffold Selection

To modulate ArM cofactor properties and catalysis, a protein scaffold must possess sufficient space to accommodate the cofactor(s) of interest while still allowing for beneficial interactions with the cofactor and potential substrates.³⁶ The large size and octahedral geometry of metal polypyridyl complexes places significant constraints on the types of scaffolds that can be used. Our group has used *Pfu* POP as a scaffold for ArM formation via bioconjugation with bulky acridinium cofactors³⁷ and dirhodium tetracarboxylate (**2**, Figure 1b) cofactors^{38,39}. MD simulations of the latter indicate that POP can fully encapsulate **2** while arraying active site residues proximal

to it, and the high selectivity observed for a variety of reactions catalyzed by dirhodium ArMs indicates that these interactions significantly influence cofactor reactivity^{38,40}. The POP scaffold was therefore selected for construction of ArM photocatalysts.

Figure 1. Overview of ArM Photocatalyst Preparation and Characterization. A) Combining conventional photocatalysts with a protein scaffold enables modulation of the cofactors' properties through interactions with the biomolecule. B) Previously reported dirhodium^{38,39} and acridinium³⁷ cofactors.

Covalent ArM Preparation and Characterization

In analogy to the dirhodium and acridinium systems noted above (Figure 1b), strain-promoted azide-alkyne cycloaddition (SPAAC) was used to covalently link endo-[6.1.0]-bicyclononyne (BCN)-substituted Ru(bpy)₃²⁺ cofactor **1a** within POP scaffolds containing a genetically encoded azidophenylalanine (Z) residue⁴¹. Cofactor **1a** was synthesized by introducing 4-hydroxymethyl-Bpy **ii**⁴² (prepared from commercially available methyl isonicotinate **i**) onto Ru(Bpy)₂Cl₂⁴³ via ligand exchange to generate **iii** and then reacting this complex with carbonate **iv**⁴⁴ (Scheme 1). The exo isomer of the cofactor (**1b**) was also prepared since these isomers could differentially orient the Ru(Bpy)₃²⁺ fragment of **1a** within the POP active site. Furthermore, because octahedral Ru(Bpy)₃²⁺ complexes can adopt Δ and Λ configurations,⁴⁵ resolved Δ - and Λ -Ru(Bpy)₂(Py)₂ tartrate salts were used to prepare the endo/ Λ , endo/ Δ , exo/ Λ , and exo/ Δ cofactors (**1c-1f**, respectively, Supplementary Figure 1).

POP-Z variants were then generated by genetically encoding the Z residue at active site positions 53, 99, 326, 338, and 477 to explore the effects of cofactor linkage site on ArM function. All variants expressed in good yields (~25-100 mg/L), and intact ESI-MS confirmed the expected masses of the proteins (Supplementary Figure 2). SPAAC conditions developed for dirhodium ArMs were used to link **1a** to these scaffolds,⁴⁰ and intact ESI-MS again confirmed the expected masses of the ArMs (Figure 2A and Supplementary Figure 2). Notably, while bioconjugation of **2** is typically complete within 10 min, 6-18 hours of incubation was required to achieve >99% bioconjugation of **1a** to all POP-Z variants examined except for POP_{WT}-Z₅₃, which was also complete within 10 min (Supplementary Figure 3).

Scheme 1. Synthesis of BCN-substituted $\text{Ru}(\text{Bpy})_3^{2+}$ cofactor **1a** (*p*NP = *p*-nitrophenyl ester). (a) **i**, H_2O_2 , acetic acid, 70°C , O/N (92 %); (b) pyridine N-oxide, $\text{Pd}(\text{OAc})_2$, K_3CO_3 , 2-bromopyridine, $[\text{P}(\text{tBu})_3\text{H}]\text{BF}_4$, toluene, RT $\rightarrow 120^\circ\text{C}$, O/N (54 %); (c) 4-methoxycarbonyl-2-(pyridin-2-yl)pyridine N-oxide, Pd/C , EtOH, H_2 , RT, 4 hours (95 %); (d) 4-methoxycarbonyl-2-(pyridin-2-yl)pyridine, LiBH_4 , THF, 0°C (1 hour) $\rightarrow$ RT, 12 hours (83 %); (e) 2-(pyrid-2-yl)-4-hydroxymethyl-pyridine **ii**, *cis*- $\text{Ru}(\text{Bpy})_2\text{Cl}_2$, EtOH, reflux (O/N) $\rightarrow$ RT, NH_4PF_6 , H_2O (54%); (f) **iii**, NaH, ACN, RT, 5 min $\rightarrow$ **iv**, THF, RT, 1 hour $\rightarrow$ acetone, NH_4PF_6 , H_2O (84%).

A variety of spectroscopic methods were next used to provide insight into binding of **1a-f** within the different POP-Z variants. The λ_{max} values in the absorbance and emission spectra of the cofactors did not change significantly upon bioconjugation (Supplementary Figure 4-5), consistent with the fact that the orbital energies of $\text{Ru}(\text{Bpy})_3^{2+}$ and related complexes are generally not sensitive to changes in solvent/environment⁴⁶. The luminescence lifetimes of the ArMs were also evaluated since it is known that the lifetime of luminophores like $\text{Ru}(\text{Bpy})_3^{2+}$ increases in more hydrophobic environments,^{30,47,48} such as that expected within the POP active site, and substantial increases compared to the free complexes were observed (Table 1).

Table 1. Luminescence Lifetimes of Covalent ArMs

Entry	Scaffold	Cofactor	Stereochemistry	Lifetime (ns)
1	N/A	3a	racemic / n/a	573
2	N/A	^a 1a	racemic / endo	530
3	POP _{WT} -Z ₅₃	1a	racemic / endo	765
4	POP _{WT} -Z ₉₉	1a	racemic / endo	791
5	POP _{WT} -Z ₃₂₆	1a	racemic / endo	761
6	POP _{WT} -Z ₃₃₈	1a	racemic / endo	773
7	POP _{WT} -Z ₄₇₇	1a	racemic / endo	837
8	POP _{WT} -Z ₅₃	1b	racemic / exo	725
9	POP _{WT} -Z ₅₃	1c	Λ / endo	737
10	POP _{WT} -Z ₅₃	1d	Δ / endo	715
11	POP _{WT} -Z ₅₃	1e	Λ / exo	726
12	POP _{WT} -Z ₅₃	1f	Δ / exo	715

^aMeasurements performed in 0.5% ACN. All other measurements were performed in MQ H_2O .

Finally, CD spectroscopy was used to more directly interrogate possible interactions between **1a-f** and the different protein scaffolds (Figure 2C and Supplementary Figure 6). Optically-active chiral molecules like $\text{Ru}(\text{Bpy})_3^{2+}$ differentially absorb left- and right-circularly polarized light,⁴⁹ and

the resulting Cotton effects (i.e. CD signals near absorbance bands) can be used to analyze optical activity of metal complexes bound to a protein host⁵⁰. While the apo proteins were spectroscopically silent above 250 nm, positive and negative Cotton effects near 300 nm were observed for ArMs prepared with enantiopure cofactors (Figure 2C). Similar Cotton effects were observed for POP_{WT}-Z₅₃-**1a** (endo/racemic) and POP-Z₅₃-**1b** (exo/racemic). These observations indicate that POP_{WT}-Z₅₃ possesses sufficient affinity for the Ru(Bpy)₃²⁺ fragment of **1c/1e** (Λ isomers) to enable partial resolution of racemic **1a** during bioconjugation with an excess of this cofactor (2.75 equivalents). This apparent affinity explains the increased rate of bioconjugation for POP_{WT}-Z₅₃. Consistent with these observations, docking simulations of **1e** revealed that the Ru(Bpy)₃²⁺ fragment of **1e** preferred to bind in a pocket that projected the BCN fragment toward residue 53 but not any of the other residues in the POP-Z variants examined (Figure 2D).

Figure 2. Covalent ArM Characterization. a) Intact ESI-MS characterization of POP_{WT}-Z₅₃ before (theoretical: 71,947 Da) and after SPAAC reaction (theoretical: 72,723 Da) with cofactor **1a** (endo/racemic). b) Excitation ($\lambda_{\text{exc}} = 260\text{--}610\text{ nm}$, $\lambda_{\text{em}} = 620\text{ nm}$) and emission ($\lambda_{\text{exc}} = 450\text{ nm}$, $\lambda_{\text{em}} = 460\text{--}900\text{ nm}$) spectra (upper) for POP_{WT}-Z₅₃-**1a** ArM (50 μM). Luminescence lifetime ($\lambda_{\text{exc}} = 450\text{ nm}$, $\lambda_{\text{em}} = 620\text{ nm}$) decay (lower) obtained for POP_{WT}-Z₅₃-**1a** ArM (50 μM). c) Circular dichroism spectra of various POP_{WT}-Z₅₃ ArMs (20 μM). d) Docking of cofactor **1e** (exo/ Λ ; crimson) with POP_{WT} (grey) scaffold using AutoDock Vina⁵¹. The alkyne functional group on the BCN

moiety is oriented towards residue M53, and not towards other residues (e.g. Y326 and R338) targeted for incorporation of azidophenylalanine.

Non-Covalent ArM Preparation and Characterization

To further explore the apparent binding of the $\text{Ru}(\text{Bpy})_3^{2+}$ fragments of **1a-f** within POP, the affinity of POP_{WT} toward a variety of substituted $\text{Ru}(\text{Bpy})_3^{2+}$ complexes was examined. Luminescence lifetime titrations^{52–54} established that $\text{Ru}(\text{Bpy})_3^{2+}$ (**3a**) binds to POP_{WT} with a K_d of $28 \pm 9 \mu\text{M}$. Control experiments with BSA indicate that **3a** possesses minimal non-specific binding affinity towards a similarly-sized globular protein (Supplementary Figure 7). The unexpected observation of **3a** binding to POP thus provides a means to readily characterize the effects of changes in both protein and cofactor structure on binding affinity and photophysical properties.

We first aimed to increase the affinity of POP for **3a** via targeted mutations within the POP active site (Figure 3a). Several active site arginine and lysine residues (R55, R198, K255, and R338) that could have repulsive interactions with positively charged $\text{Ru}(\text{Bpy})_3^{2+}$ derivatives were mutated to alanine in POP_{G99A} . Active site tyrosine and tryptophan residues (W142 and Y326) were also mutated to alanine to avoid potential oxidation by $\text{Ru}(\text{Bpy})_3^{3+}$ derivatives, which could be accessed via oxidative quenching with an exogenous oxidant,^{55,56} giving POP_{Neut} . Residues 99, 142, and 326, all projecting into the active site, were also mutated to aspartic acid in the POP_{Neut} scaffold to generate variant POP_{Neg} . With K_d values of $1.7 \pm 0.7 \mu\text{M}$ and $1.1 \pm 1.7 \mu\text{M}$ for POP_{Neut} and POP_{Neg} , respectively, both variants had improved affinities for **3a** (Figure 3). The apparent dissociation constant for POP_{Neg} is only an upper limit due to experimental restrictions arising from the high affinity of the interaction (see Supplementary Information for details)⁵⁷.

To confirm that **3a** was binding in the active site of POP, a competition experiment was conducted using this complex and the known covalent POP inhibitor, Z-Pro-Prolinal (ZPP)⁵⁸. Increasing inhibitor concentrations resulted in lower lifetime values, though the values were still significantly greater than the free cofactor, consistent with reduced affinity of the covalently modified active site for **3a** (Supplementary Figure 8). In a series of complementary experiments inspired by the improved binding of **3a** to POP_{Neut} and POP_{Neg} relative to POP_{WT} , the ionic strength of the medium was increased to better solvate dicationic **3a** and thus disfavor binding in the POP active site (Figure 3b and Supplementary Figures 9-10). Increased concentrations of KCl and various other salts/buffers significantly perturbed binding of **3a** to POP_{Neg} . Furthermore, non-covalent ArMs treated with different aqueous salts during spin filtration retained no **3a** while those treated only with water retained a significant amount of cofactor.

We next explored the extent to which the chirality of $\text{Ru}(\text{Bpy})_3^{2+}$ derivatives and substitution on the Bpy ligands affects cofactor binding. As noted above, docking simulations suggest that BCN-substituted cofactor **1c** can bind to a pocket in the hinge region of the POP active site. Analogous simulations using $\text{Ru}(\text{Bpy})_3^{2+}$ complexes **3b** and **3c** (Λ and Δ configurations, respectively) indicate that this pocket could accommodate both cofactor enantiomers. These simulations also indicate that the 4,4' hydrogens of one Bpy ligand extended into this pocket with enough room to accommodate modifications at these positions (Figure 3c). Consistent with the docking simulations, comparable binding of enantiopure $\text{Ru}(\text{Bpy})_3^{2+}$ complexes **3b** and **3c** to POP_{WT} (K_d values of $30 \mu\text{M}$ and $37 \mu\text{M}$, respectively) was observed (Table 2 and Supplementary Figure 11). Racemic complexes displaying 4,4'- CO_2^- , $-\text{CH}_2\text{CO}_2^-$, $-\text{CH}_2\text{OH}$, $-\text{tBu}$, $-\text{NH}_2$, and $-\text{CH}_2\text{NH}_3^+$ substituents (**3d-i**) were prepared, and luminescence lifetime titrations (Figure 3d and Supplementary Figure 12) revealed minor differences in affinity towards POP_{WT} , except for **3g** and **3i**, which bind 1.4- and 1.5-fold tighter than **3a**, respectively (Table 2).

We also observed different changes in the luminescence lifetime (Table 2) of the free vs. bound cofactors (Δ_{lifetime}), and in many cases, significant increases in the luminescence intensity of the cofactor (Supplementary Figure 13). Analogous binding experiments were also performed using POP_{Neg} (Supplementary Figure 14-15). While similar trends were observed, there were notable differences between acid-substituted complex binding to the protein variants, as **3d** had a moderately lower affinity for the POP_{Neg} scaffold ($K_d = 185 \mu\text{M}$) compared to POP_{WT} ($K_d = 30 \mu\text{M}$), perhaps due to electrostatic repulsion between the anionic cofactor and the aspartate residues (i.e. 99D, 142D, and 326D) in the active site of POP_{Neg}.

Figure 3. Modulating Non-Covalent ArM Formation through Protein and Cofactor Engineering. a) Luminescence lifetime titrations ($\lambda_{\text{exc}} = 450 \text{ nm}$, $\lambda_{\text{em}} = 620 \text{ nm}$) demonstrate binding of POP variants to **3a**. b) **3a** binding to POP_{Neg} is disrupted by increasing concentrations of KCl. c) Docking simulations between **3b** (gold) and **3c** (blue) with POP_{Neg} (grey) reveal potential binding in the enzyme's active site. The 4,4'-hydrogens (white) of one ligand project into the hydrophobic pocket. d) Luminescence lifetime titrations ($\lambda_{\text{exc}} = 450 \text{ nm}$, $\lambda_{\text{em}} = 620\text{-}650 \text{ nm}$) demonstrate binding of POP_{WT} and POP_{Neg} to various 4,4'-substituted ruthenium complexes. Plotted data points represent single measurements and error bars represent standard deviations resulting from single exponential tail fitting of decay data. Data were fit with a quadratic binding equation to give curves shown (Eq. 1 in the Supplementary Information).

Table 2. Binding Affinities and Luminescence Lifetimes of Non-Covalent ArMs

Entry	Variant	Cofactor	R	K _d (μM)	Δlifetime (ns)
1	POP _{WT}	3a	H	28.3 ± 8.8	96
2	POP _{Neut}	3a	H	1.65 ± 0.71	166
3	POP _{Neg}	3a	H	^a 1.08 ± 1.70	183
4	POP _{WT}	3b	H	30.1 ± 8.2	110
5	POP _{WT}	3c	H	36.8 ± 11.1	86
6	POP _{WT}	3d	-CO ₂ ⁻	29.5 ± 9.8	35
7	POP _{Neg}	3d	-CO ₂ ⁻	185 ± 20	125
8	POP _{WT}	3e	-CH ₂ CO ₂ ⁻	30.8 ± 10.1	60
9	POP _{WT}	3f	-CH ₂ OH	30.8 ± 10.4	147
10	POP _{WT}	3g	- <i>t</i> Bu	19.6 ± 5.7	500
11	POP _{WT}	3h	-NH ₂	^b NA	NA
12	POP _{WT}	3i	-CH ₂ NH ₃ ⁺	18.7 ± 2.0	142

^aThis value is only an estimated upper bound of affinity. The binding data exhibited characteristics consistent with being in the “titration” regime⁵⁷. Refer to the Supplementary Information for more details. ^bLow luminescence intensities precluded accurate determination of the lifetime of this complex at reasonable concentrations (i.e. << [POP]).

POP ArMs for Photocatalysis

Having established two approaches to link different photocatalysts to POP scaffolds, we next sought to evaluate the catalytic properties of these systems. Yoon has reported two mechanistically distinct reactions involving catalytic quantities of Ru(Bpy)₃²⁺ and a Brønsted acid co-catalyst. One of these involves 5-exo-trig reductive cyclization of dienones to generate 5-membered rings via electron transfer (Scheme 2a);⁵⁹ the second involves [2+2] cycloaddition between C-cinnamoyl imidazoles and different alkenes to give cyclobutanes via energy transfer (Scheme 2b)^{60,61}. Both reactions worked well in 10% aqueous ACN. Brønsted acid co-catalysts were not required in this medium, though a slightly acidic pH was optimal (Supplementary Fig. 16) for the cycloaddition (25 mM MES pH 6.0; pK_a of related N-methyl imidazole ~ 7.0), and ascorbate served as a water-soluble reductant for the reductive cyclization reaction (Supplementary Table 1-2).

Scheme 2. a) Photoreductive cyclization reaction of dienone **4** and b) [2+2] photocycloaddition of cinnamoyl imidazole **6** and 4-methoxystyrene **7**. Yields were determined by UHPLC relative to an internal standard, 1,3,5-trimethoxybenzene (TMB). Reactions were performed in triplicate (n=3) and are reported as averages with standard deviations.

The optimized conditions for both reactions were used to screen the reactivity and selectivity of a small panel of ArMs generated from different POP scaffolds. Only covalent ArMs were evaluated since ascorbate and MES, like other salts and buffers (*vide supra*), displaced the complex from non-covalent ArMs (Supplementary Figure 17). Reactions were conducted in an inert atmosphere wetbox containing a custom-built LED photoreactor compatible with 96-well plates to enable consistent irradiation samples (Supplementary Figure 18). The desired products, **5** and **8**, were observed in all cases, and higher conversions were generally observed for ArM-catalyzed reactions relative to the free cofactor (Scheme 2, Supplementary Tables 3-4). Furthermore, increased rates of reaction were observed relative to Ru(Bpy)₃²⁺ with a 2-fold increase for reaction with **4** (0.21 μ M/min vs. 0.41 μ M/min for **3a** and POP_{Neg}-Z₅₃-1f, respectively; Supplementary Figure 19) and a 2.6-fold increase for reaction with **6** (4.7 μ M/min vs. 12.0 μ M/min for **3a** and POP_{WT}-Z₅₃-1d, respectively; Supplementary Figure 20).

Discussion

This study describes the formation of ArMs via covalent and non-covalent incorporation of Ru(II) polypyridine visible light photocatalysts into engineered POP variants. While our selection of metal polypyridine photocatalysts was motivated by their stability and the diversity of reactions that they catalyze,⁵ their large size presents a challenge for ArM design. Privileged scaffolds like myoglobin⁶² or LmrR⁶³ that bind a variety of planar complexes, for example, would likely not accommodate bulky cofactors like **1a-f**. More open scaffolds like streptavidin do not fully encapsulate anchored catalysts,⁶⁴ so their ability to modulate cofactor properties and control reactivity is limited (though fused lid structures such as that recently reported for streptavidin could correct this issue)⁶⁵. We previously established that *Pfu* POP is a versatile scaffold for ArM formation⁶⁶ and that directed evolution can be used to engineer selective ArMs for a variety of dirhodium-catalyzed carbene addition and insertion reactions^{38,40}. We have also used this scaffold to develop an artificial enzyme containing an acridinium cofactor (Figure 1b) for visible light photoredox catalysis.³⁷ This system catalyzes sulfide oxidation via SET, but no selectivity was obtained, presumably due to poor substrate binding within the active site. Rapid photobleaching of the acridinium cofactor limits the potential of this system even if selectivity could be achieved.

Preliminary efforts to develop POP ArM photocatalysts focused on cofactor **1a** (Figure 1a), which contains a [6.1.0]-bicyclononyne anchor for SPAAC to proteins containing a genetically encoded azidophenylalanine residue. SPAAC proceeded efficiently, giving >99% bioconjugation in all cases. Interestingly, the linkage site strongly influenced the rate of SPAAC, and POP_{WT}-Z₅₃ was even able to partially resolve racemic cofactor **1a** to form an ArM enriched in the Λ cofactor enantiomer. Similar stereoselective bioconjugation of ruthenium complexes to proteins has been described previously.³⁰⁻³³ For example, Salmain showed that Michael addition of the active site cysteine of papain to maleimide-substituted Ru(II) complexes proceeded stereoselectively and that substituents on these complexes affect bioconjugation rates.³⁰ Tiede later showed that bioconjugation of PpcA via cysteine alkylation also led to cofactor resolution, and differences in stereoselectivity based on bioconjugation site were noted.³¹ Together, these studies provide precedent for the site-dependent bioconjugation rate acceleration observed in the current study, but both involved relatively small scaffolds that left their cofactors solvent exposed. POP is large enough to accommodate both the cofactor and potential substrate(s) in its hydrophobic active site, and secondary Ru(II) polypyridine binding provides a means to reduce cofactor movement, which has been found to improve the activity and selectivity of ArMs generated from other scaffolds^{67,68}.

Although covalent cofactor bioconjugation allows for complete removal of free cofactor and associated background spectroscopic signals or catalysis,⁴⁰ it necessitates the incorporation of bioorthogonal linkage sites into both cofactor and scaffold⁴¹. *High affinity* non-covalent cofactor

binding eliminates these issues and inherently provides a direct interface between active site residues and the cofactor to modulate cofactor properties.⁶⁹ Consistent with the apparent binding of the Ru(II) fragment of cofactor **1a**, POP exhibited Ru(Bpy)₃²⁺ binding affinities in the μ M range. Reduced luminescence lifetimes for this system in the presence of a known covalent inhibitor of POP, Z-Pro-Prolinal,⁵⁸ and negligible binding to BSA highlighted the unique affinity of the POP active site for Ru(Bpy)₃²⁺. This fortuitous discovery provides a starting point to develop non-covalent ArM photocatalysts in which both cofactor and scaffold can be tuned to modulate cofactor binding, photophysical properties, and reactivity.

Initial non-covalent ArM engineering efforts focused on further improving cofactor binding. Given that Ru(Bpy)₃²⁺ is dicationic, several positively charged residues in the POP active site were mutated to Ala or Asp to create POP_{Neut} and POP_{Neg}, respectively, with the goal of improving electrostatic scaffold/cofactor complementarity. This approach mirrors analogous charge complementation in both natural proteins (e.g. cytochrome f in photosystem I)^{70–72} and synthetic systems (e.g. methyl viologen in streptavidin)²⁹. Non-covalent binding in POP_{Neut} and POP_{Neg} increases substantially, and K_d values approaching the nM regime are observed for Ru(Bpy)₃²⁺ binding. The electrostatic nature of cofactor binding in these systems is supported by control experiments in which added KCl and other salt solutions that could better solvate the cofactor significantly reduce binding of **3a** even to POP_{Neg}. Cofactor substitution was also found to modulate binding. Docking simulations were used to guide the installation of substituents on the 4 and 4' positions of one Bpy ligand that could interact with the protein via different mechanisms (e.g. hydrophobic effect, hydrogen bonding, ion pairing, etc.). *t*Bu-substituted complex **3g** was found to bind better than the homoleptic complex **3a**, highlighting the importance of the hydrophobic effect for cofactor binding.

The Ru(II) polypyridine ArMs developed in this study also provide a unique platform to tune and study the photophysical properties and reactivity of visible light photocatalysts. Both covalent and non-covalent ArMs exhibit increased luminescence lifetimes and intensities relative to free cofactors. These changes were dependent on the identities of the cofactor and scaffold. For example, while the average increase in the luminescence lifetime upon non-covalent binding to POP_{WT} was around 100 ns for most cofactors, the lifetime of **3g** was increased by 500 ns (from 486 ns to 986 ns), similar to the lifetime of Ru(Bpy)₃²⁺ in deoxygenated acetonitrile (930 ns)^{5,8}. Similar enhancements in different Ru(II) polypyridine-protein conjugates have been ascribed to Ru(II) polypyridine binding in hydrophobic sites that can shield the complexes from luminophore-quenching oxygen.³⁰ Both of these mechanisms could be operative for POP ArMs given the large, hydrophobic Ru(II) polypyridine binding pocket within the POP active site (Figure 3c). The site of cofactor attachment and electrostatic complementation were also observed to modulate the luminescence of covalent cofactors. For instance, ArMs constructed by bioconjugating **1a** at different active site residues had lifetimes ranging from 761 ns to 837 ns (Table 1), and the addition of an anionic carboxylate group (**3d**) limited the increase in lifetime to only 35 ns upon non-covalent association with POP_{WT} vs. 125 ns for POP_{Neg}, highlighting the dependence of cofactor properties on the local protein environment. The importance of linker design/placement in synthetic systems was recently demonstrated in a systematic study focused on the effects of ligand modification (i.e. length and chiral configuration) and chromophore placement on the photophysics of Sulforhodamine B.⁷³ While we only investigated one linker in this study, the rigidity, length, and stereochemistry of the BCN moiety could be tuned to further control the nature of protein-cofactor interactions.

Finally, covalent ArM photocatalysts exhibited promising activity towards two mechanistically distinct transformations. In the first, 5-exo-trig reductive cyclization of a dienone (e.g. **4**) generates a cyclopentane (e.g. **5**) via SET using a sacrificial reductant.⁵⁹ In the other model reaction, [2+2]

cycloaddition between a C-cinnamoyl imidazole (e.g. **6**) and an electron-rich styrene like 4-methoxystyrene yields a cyclobutane (e.g. **8**).^{60,61} In both cases, enhanced yields and rate acceleration compared to the free cofactor were observed under optimized aqueous conditions. These improvements could result from increased local concentration of both substrate and photocatalyst within the ArM active site relative to solution. The increased luminescence lifetime of ArM photocatalysts could also improve yields since longer-lived excited states increase the probability of the catalyst encountering substrates in a bimolecular reaction. Small differences in the diastereomeric ratios (d.r.) for ArM- and Ru(Bpy)₃²⁺-catalyzed [2+2] cycloaddition were observed (1.2 and 1.5, respectively; Supplementary Figure 21), suggesting that the protein scaffold could modulate reaction stereoselectivity and providing a starting point for further engineering efforts.

Conclusion

In this study, we developed a series of covalent and non-covalent ArMs using metal polypyridyl cofactors and a prolyl oligopeptidase scaffold. Modifications to both the scaffold and the cofactor were used to increase cofactor binding affinity and extend its luminescence lifetime, an important metric when assessing the potential of a photocatalyst. The ArM photocatalysts exhibited good activity toward reactions that proceed via SET (reductive cyclization) and ET ([2+2] photocycloaddition) under optimized aqueous reaction conditions. Moreover, the yields of the desired products and rates of reactions were enhanced for ArM-catalyzed reactions compared to Ru(Bpy)₃²⁺ under aqueous conditions. The ability of POP to bind and modulate the properties of different Ru(II) cofactors suggests that ArM photocatalysts could serve as a general platform for the further development of diverse catalysts with tunable photophysical properties and reactivity.

Supplementary Information

Electronic supplementary information (ESI) available: Supplemental figures, detailed procedures and results for all reported experiments, and data for compound characterization.

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