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Preparation, Characterization, and Oxygenase Activity of a Photocatalytic Artificial Enzyme

Yifan Gu, Ken Ellis-Guardiola, Poonam Srivastava, and Prof. Jared C. Lewis

Department of Chemistry, University of Chicago, 5735 S. Ellis Ave., Chicago, IL 60637, USA

Abstract

A bicyclo[6,1,0]nonyne-substituted 9-mesityl-10-methyl-acridinium cofactor was prepared and covalently linked to a prolyl oligopeptidase scaffold containing a genetically encoded 4-azido-L-phenylalanine residue in its active site. The resulting artificial enzyme catalyzed sulfoxidation when irradiated with visible light in the presence of air. This reaction proceeds via initial electron abstraction from the sulfide within the enzyme active site, and the protein scaffold extended the fluorescence lifetime of the acridium cofactor. The mode of sulfide activation and placement of **5** in POP-ZA₄-**5** make this artificial enzyme a promising platform for developing selective photocatalytic transformations.

Keywords

artificial enzyme; photocatalyst; oxygenase; prolyl oligopeptidase; unnatural amino acid

Chemists have long sought to design catalysts that mimic the ability of natural photosynthetic systems to harvest visible light photons and use the energy from photon absorption to drive chemical reactions.^[1] The protein complexes that evolved to accomplish these tasks, photosystems II and I, catalyze light-driven water oxidation and transmembrane electron transport, respectively.^[2] Photosynthetic organisms use the electrons transported by photosystem I (PSI) to reduce NADP⁺ to NADPH, but a number of biohybrid systems capable of harnessing these electrons to reduce protons to H₂ for solar fuels applications have been developed by linking PSI to synthetic catalysts.^[3] These systems show that the unique photophysical properties imparted to natural chromophores embedded within the PSI reaction center protein scaffolds^[4] can be exploited for non-natural chemical reactions.

Recently, an alternative biohybrid catalyst for light-driven proton reduction was created via bioconjugation of a hydrogen-evolving diiron complex to a protein scaffold lacking any bound chromophores.^[5] The photosensitizer [Ru(bpy)₃]²⁺ was added to a solution of the biohybrid to enable intermolecular electron transfer to the diiron complex upon irradiation with visible light and catalytic proton reduction in the presence of ascorbate. Prior to this work, Gray^[6] and Cheruzel^[7] had demonstrated that electrons supplied to a cytochrome P450 BM3 variant by a covalently linked [Ru(bpy)₃]²⁺ derivative upon irradiation with

Correspondence to: Jared C. Lewis.

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visible light could be used to drive hydroxylation of organic molecules, illustrating the potential of light-driven biohybrid systems to catalyze organic transformations.

Photosensitizers, including $[\text{Ru}(\text{bpy})_3]^{2+}$ and a range of other coordination complexes and organic dyes, are themselves used as catalysts for growing family organic transformations.^[1b] For example, 9-Mesityl-10-methylacridinium ($\text{Acr}^+\text{-Mes}$) perchlorate has been shown to catalyze a range of heteroatom, arene, and olefin functionalization reactions upon irradiation with visible light ($\lambda > 450 \text{ nm}$).^[8] This reactivity arises from the oxidizing properties of this species in the excited state ($E_{\text{red}} = +1.88 \text{ V vs SCE, PhCN}$).^[9] Extensive evidence suggests that $\text{Acr}^+\text{-Mes}$ is photoexcited to the singlet excited state, $^1\text{Acr}^{+*}\text{-Mes}$, which undergoes intramolecular electron transfer to form $\text{Acr}^\bullet\text{-Mes}^{+*}$, the proposed active oxidant.^[9] We hoped that incorporating an $\text{Acr}^+\text{-Mes}$ -based cofactor into a protein scaffold could generate a photocatalytic enzyme for organic oxidation reactions. While a wide range of organic and organometallic cofactors have been incorporated into protein scaffolds to create artificial enzymes,^[10] no systems in which a visible light photocatalyst that acts directly on an organic substrate is linked to a protein have been reported. We envisioned that such a system could be used to modulate the photophysical properties of visible light photocatalysts such as $\text{Acr}^+\text{-Mes}$ in analogy to natural systems^[4] and ultimately impart selectivity to reactions catalyzed by these species. As a first step towards these goals, we describe the preparation and detailed characterization of an $\text{Acr}^+\text{-Mes}$ -based artificial enzyme that catalyzes sulfoxidation when irradiated with visible light in the presence of air.

We recently reported that bicyclo[6,1,0]nonyne (BCN)-substituted cofactors could be covalently linked to into *p*-azido-L-phenylalanine (Z) substituted protein scaffolds via strain-promoted azide-alkyne cycloaddition (SPAAC).^[11] This bioconjugation method allows rapid, site-selective incorporation of cofactors into protein scaffold. To explore the possibility of incorporating $\text{Acr}^+\text{-Mes}$ cofactors into proteins, we prepared BCN-substituted $\text{Acr}^+\text{-Mes}$ **5** (Scheme 1). TBS protection of alcohol **1** followed by lithiation and addition to *N*-methylacridone, in analogy to the synthesis of $\text{Acr}^+\text{-Mes}$,^[12] provided alcohol **3**. Reaction of this alcohol with carbonate **4** provided **5**.

We selected a prolyl oligopeptidase (POP) from *Pyrococcus furiosus* (*Pfu*) as a scaffold for bioconjugation of **5** (Scheme 2).^[13] Because the structure of *Pfu* POP has not been solved, a previously reported homology model^[14] of this enzyme was used to guide our engineering efforts. This model suggested that *Pfu* POP possesses a large active site cavity within a β -barrel domain that is ideally suited for cofactor enclosure (Figure. 1a). This unique structure and the high stability of *Pfu* POP made it an attractive candidate for artificial enzyme formation, despite the lack of crystal structure. As in our initial report of artificial enzyme formation using SPAAC,^[11] an amber codon was introduced into the POP gene^[15] to replace the catalytically active serine (S477) with a Z residue (Z477). Four alanine mutations (E104A, F146A, K199A, D202A) were introduced to open a small pore in the POP structure^[16] to allow cofactor entry into the active site. A codon optimized POP gene was used as a template for genetic manipulation, and the resulting scaffold, POP-ZA₄, was expressed in high yield in *E. coli* with essentially quantitative Z incorporation. Bioconjugation of POP-ZA₄ using **5** was readily monitored using SDS-PAGE followed by UV visualization, and the expected mass of POP-ZA₄-**5** was confirmed using HR-ESI MS

(Figure. 1b/c). Moderate bioconjugation yields (ca. 50%) were obtained due in part to decomposition of the Z azide to the corresponding aniline.^[17]

Acr⁺-Mes was also prepared using literature methods^[12] and used to evaluate the efficiency of POP-ZA₄-**5** catalysis.^[18] While trace conversion was observed for several olefin addition reactions^[11] catalyzed by Acr⁺-Mes in aqueous solution irradiated with visible light, aerobic sulfoxidation^[19] of thioanisole proceeded with relatively high and consistent yields in both acetonitrile and acetonitrile/buffer (Tris or phosphate) solutions (Table 1, entries 1 and 2). POP-ZA₄-**5** also catalyzed this reaction with modest conversion (entry 3), demonstrating for the first time that a photocatalytic artificial enzyme could directly catalyze an organic transformation. The artificial enzyme tolerated up to 20% v/v acetonitrile, but higher co-solvent concentrations led to extensive enzyme precipitation over the course of the reaction. No activity was observed in the absence of visible light or using POP lacking cofactor **5** (entries 4 and 5), and in no cases was over oxidation (sulfone) observed. A clear trend favoring sulfoxidation of electron rich thioanisoles was observed (entries 3 and 6–8), and ethyl phenyl sulfide also underwent sulfoxidation (entry 9). The sulfoxide products of these reactions were isolated as racemic mixtures, and the yields (entries 2 and 3) and rates (Fig. S6) for reactions catalyzed by POP-ZA₄-**5** were generally lower than those catalyzed by **5**. Analogous problems are often observed for initial artificial enzyme constructs,^[10] including the aforementioned photocatalytic hydrogen evolution system,^[5] as a result of suboptimal active site configurations. Nonetheless, these systems offer unique starting points for engineering efforts to improve catalytic efficiency.^[20]

Photocatalytic oxygenation of sulfides^[22] and phosphines^[21] has been proposed to proceed via two distinct, often substrate dependent, mechanisms^[19] that are initiated by photocatalysts via either (a) ¹O₂ formation or (b) electron abstraction from the organic substrate. This distinction is important for the prospect of engineering selective artificial enzymes since the substrate would more likely be located within the protein scaffold during the oxygenation process in the latter case (b). While most reports indicate that this pathway is typically operative for sulfoxidation of thioanisoles,^[19] we wished to establish that this was indeed the case for POP-ZA₄-**5** since in the former case (a), the artificial enzyme could simply serve as a source of ¹O₂.^[22]

A series of experiments previously used to distinguish these mechanisms^[19] were therefore carried out. We observed no inhibition of POP-ZA₄-**5** catalyzed sulfoxidation of thioanisole in the presence of DABCO (0.05–0.2 equiv.), a known ¹O₂ quencher, and no reaction of diphenyl sulfide, which is readily converted to the corresponding sulfone using ¹O₂ sensitizers.^[23] In addition, increased concentrations of 4-methoxy thioanisole led to increased conversion of this substrate via sulfoxidation (Figure. 2). This is contrary to what is observed for sulfoxidation reactions proceeding via ¹O₂ mechanisms since the excess sulfide leads to ¹O₂ quenching and thus decreasing conversion with increasing sulfide concentrations.^[19] Together, these observations suggest that ¹O₂ is not involved in POP-ZA₄-**5** catalyzed sulfoxidation of thioanisoles. Analogous results were obtained for reactions catalyzed by **5**, indicating that the POP scaffold did not alter the reaction mechanism.

The photophysical properties of Acr⁺-Mes have been extensively studied^[9](and debated^[24]) using a range of spectroscopic methods, and significant medium effects on the fluorescence emission and lifetime of acridinium dyes have been reported^[25]. To determine if the POP scaffold has *any* impact on the properties of the Acr⁺-Mes moiety, we also investigated the photophysical properties of POP-ZA₄-**5**. We observed no significant difference in photobleaching rates and only minor differences between the absorbance and emission spectra of Acr⁺-Mes and POP-ZA₄-**5** (Figure. 2b, S1), but several notable differences in the fluorescence lifetimes of these species were apparent. First, a modest reduction in the fluorescence lifetime of Acr⁺-Mes was observed in the presence of air and further reduction occurred in aqueous solution, the conditions used for photocatalysis (Table 2, entries 1–3). Under these same conditions, however, a longer lifetime was observed for POP-ZA₄-**5** (entry 4). Very similar trends in the fluorescence lifetimes of [1,3]dioxolo[4,5-*f*][1,3]benzodioxole (DBD) dyes have also been observed^[26] and used to probe conformational changes in maltose ATP-binding cassette (ABC) transporter.^[27] Specifically, the fluorescence lifetime of a maleimide-substituted DBD dye linked to a cysteine mutant of the MalF subunit of the MalFGK₂ complex increased from 5 ns to 6.3 ns when MalF was bound with vanadate, which locks the enzyme in a conformation that is proposed to generate a more hydrophobic environment^[28] around the DBD dye. A similar increase in the fluorescence lifetime of Acr⁺-Mes in POP-ZA₄-**5** (Table 2, entry 4) relative to that in solution (entry 3) or on the outside of the protein (POP-Z37-**5**, entry 5) suggests that **5** resides within the hydrophobic POP active site of POP-ZA₄-**5**. While obviously far from the dramatic effects of PS proteins on their associated chromophores, these results provide evidence that POP can influence the photophysical properties of bioconjugated cofactors.

This communication describes the synthesis of a BCN-substituted Acr⁺-Mes photocatalyst (**5**) that can be linked to proteins containing a genetically encoded Z residue. Linking **5** to an engineered POP scaffold led to the formation of the first reported photocatalytic artificial enzyme to act directly on an organic substrate. A series of mechanistic experiments provided evidence that sulfoxidation catalyzed by POP-ZA₄-**5** (and **5** itself) proceeds via initial electron abstraction from the sulfide, rather than via ¹O₂. Fluorescence lifetime measurements were consistent with the cofactor being located within the hydrophobic POP active site. The apparent mode of sulfide activation and placement of **5** in POP-ZA₄-**5** make this artificial enzyme a promising platform for developing selective photocatalytic transformations, and further studies are underway to achieve this goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Qualitative model of POP-ZA₄-5 constructed in PyMOL by fusing a DFT-optimized structure of the cofactor to a homology model of the POP-scaffold. Sites of alanine mutations are shown in magenta. (inlay: expanded view of cofactor). (b) UV-Vis spectra of POP-ZA₄ (red), 5 (green), and POP-ZA₄-5 (blue). (c) ESI HRMS spectra of POP-ZA₄ (red) and POP-ZA₄-5 (blue).

Figure 2.

Sulfoxide (μmol) formed after 8 h irradiation vs. initial concentration (mM) of 4-methoxyphenyl methyl sulfide. POP-ZA₄-5 loading: 0.05 mM. Total volume: 300 μL 20% v/v ACN/Tris. Conversions ranged from 8–40%.

Scheme 1.

Synthesis of BCN-substituted 9-mesityl-10-methylacridinium cofactor 5 (*p*NP = *p*-nitrophenyl ester).

Table 1Efficiency and scope of POP-ZA₄-5 catalyzed sulfoxidation^a

Entry	R ¹ , R ²	Cat.	Yield (%) ^b
1 ^c	H, Me	Acr ⁺ -Mes ClO ₄ ⁻	90
2	H, Me	Acr ⁺ -Mes ClO ₄ ⁻	70
3	H, Me	POP-ZA ₄ -5	45
4 ^d	H, Me	POP-ZA ₄ -5	n.r.
5	H, Me	POP	n.r.
6	<i>p</i> -OMe, Me	POP-ZA ₄ -5	90
7	<i>o</i> -CO ₂ Me, Me	POP-ZA ₄ -5	20
8	<i>p</i> -NO ₂ , Me	POP-ZA ₄ -5	0
9	H, Et	POP-ZA ₄ -5	26

^aReaction performed with 2 mM sulfide, 0.1 mM catalyst in Air saturated 20% ACN/50 mM tris buffer-HCl (pH 7.4), irradiated by 450 nm LEDs at r.t.

^bGC yield.

^cin ACN.

^dwithout LEDs.

Table 2Fluorescent lifetimes of Acr⁺-Mes species

Entry	Catalyst	Solvent	Atm.	Lifetime (ns) ^a
1	Acr ⁺ -Mes	ACN	N ₂	8.19
2	Acr ⁺ -Mes	ACN	Air	6.65
3	Acr ⁺ -Mes	20% ACN/tris	Air	4.10
4	POP-ZA ₄ -5	20% ACN/tris	Air	4.92
5	POP-Z37-5	20% ACN/tris	Air	4.72

^aLifetimes were calculated using a fractional average of two exponential functions that were required to accurately model the observed fluorescence decay data (see Table S3).^[27]