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Ru-Porphyrin Protein Scaffolds for Sensing O_2

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Proteins that natively bind heme are under-utilized scaffolds for porphyrin-based tools in biology.[1,2] Porphyrins have broad applications, ranging from dyes in solar cells[3] to sensitizers for radiotherapy[4] to frameworks for catalysis.[5] Traditional methods for unnatural porphyrin incorporation into hemoproteins have limited their utility as biological tools. Harsh, denaturing conditions are typically required to remove native heme from proteins,[6,7] dramatically decreasing the number of viable protein constructs. We recently reported a novel method for incorporation of unnatural porphyrins into hemoproteins during protein expression.[8] Here we demonstrate the versatility of this expression-based method through the development of protein-based sensors, in which the native heme cofactor of different hemoprotein scaffolds has been substituted with an unnatural porphyrin for oxygen (O_2) sensing within biological contexts.

O_2 is a key metabolic indicator for profiling the physiology of tissues and cells.[9] Quenching of small molecule luminescence by O_2 is a simple, noninvasive method for imaging *in vivo* O_2 levels.[10] However, the utility of small molecules for this application is hampered by lack of targetable delivery, nonspecific binding, self-aggregation, and limitations in photophysical properties.[11,12] Incorporation of luminescent porphyrins into hemoprotein scaffolds provides a novel platform to address these issues.

Ruthenium(II) CO mesoporphyrin IX (RuMP) (Figure S1) is an ideal cofactor for protein-based sensors because it exhibits O_2-senstive phosphorescence[13] and presents a proximal axial ligation site[14] to facilitate binding to the protein scaffold. Myoglobin (Mb) and the H-NOX (Heme Nitric oxide/OXygen binding) domain from the thermophilic bacterium *Thermoanaerobacter tengcongensis* (*Tt* H-NOX) are robust proteins for RuMP sensors, as they can be readily modified with genetically encoded affinity tags and site-directed mutagenesis.[1,15] In addition, *Tt* H-NOX is stable under extreme temperatures (>70 °C).[15]

Experimental details for preparation and characterization of RuMP-substituted Mb (Ru Mb) and *Tt* H-NOX (Ru *Tt* H-NOX) are described in the Supporting Information. Briefly, RuMP was synthesized in a manner similar to published methods[14] and incorporated into Mb and *Tt* H-NOX during anaerobic protein expression. The RuMP-substituted proteins were isolated containing a stochiometric amount of porphyrin (Table S1). Indeed, further evaluation of the stability of Ru *Tt* H-NOX indicated no detectible porphyrin loss for >24 h under biological conditions (mouse plasma at 37 °C, Figure S2).

Purified Ru *Tt* H-NOX was crystallized to verify proper porphyrin insertion and preservation of the protein fold. The high-

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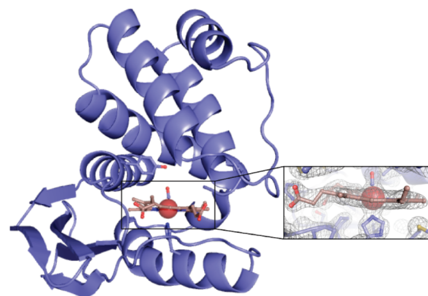

Figure 1. Crystal structure of *Tt* H-NOX containing RuMP solved at 2.00 Å resolution. Inset: $2F_o - F_c$ electron density map calculated by omitting RuMP and the proximal histidine side chain.

resolution (2.00 Å) structure of Ru *Tt* H-NOX (Figure 1, Table S2) is the first crystal structure of a Ru-porphyrin bound to a protein and demonstrates that the unnatural porphyrin maintains key contacts with surrounding heme pocket residues. These contacts include coordination of the proximal histidine to Ru and hydrogen bonding between the distal porphyrin ligand and a tyrosine residue (Figure 1, Figure S3). In fact, comparison of heme-bound *Tt* H-NOX with its Ru analogue indicates little perturbation of the protein fold (overall rmsd 1.3 Å, Figure S4).

Steady-state and time-resolved spectroscopies were employed to examine the spectral properties of RuMP bound to the protein scaffolds. UV−visible spectra for Ru *Tt* H-NOX and Ru Mb show similar Soret band features at 400 and 397 nm, respectively (Figure 2, Table 1). However, the α band at ∼550 nm for Ru Mb is split, as observed previously.[14] Steady-state emission spectra reveal a blue-shifted emission band and decreased emission quantum yield for Ru Mb as compared to Ru *Tt* H-NOX (Table 1). Time-resolved emission spectroscopy conducted to further probe the spectral features of the porphyrin−protein complexes yielded single-exponential emission decays (following 550 nm laser excitation)

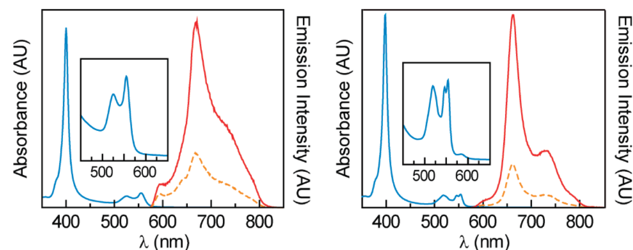

Figure 2. Steady-state absorbance (blue) and emission spectra of Ru *Tt* H-NOX (left) and Ru Mb (right) in aqueous HEPES/NaCl buffer. Emission spectra were acquired following excitation at 550 nm in the presence (orange dashed, 256 μM) and absence (red) of O_2.

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Table 1. Spectroscopic and Photophysical Properties of Ru Proteins

Ru Protein	λ_{abs} (nm) / (ε)[a]	λ_{em}[b] (nm)	Φ_{em}[d]	τ_{em}[e] (μs)
Tt H-NOX	400 (173)	668	1.7×10^{-4}	7.7 ($-O_2$)
	524 (10.7)	~734[c]		2.9 ($+O_2$)
	555 (13.9)			
Mb	397 (197)	663	4.8×10^{-5}	37.3 ($-O_2$)
	518 (12.2)	~733[c]		12.2 ($+O_2$)
	553 (13.2)			

[a] $mM^{-1}cm^{-1}$. [b] λ_{ex} = 550 nm. [c] Shoulder. [d] λ_{ex} = 550 nm, no O_2. [e] λ_{ex} = 550 nm, λ_{det} = 640 nm, no O_2 and 256 μM O_2.

under anaerobic conditions that vary widely between the two proteins (τ_o = 7.7 μs for Ru *Tt* H-NOX vs 37.3 μs for Ru Mb). Taken together, these data indicate a substantially different conformation and/or chemical environment for RuMP in Mb and *Tt* H-NOX. Indeed, the crystal structure of Mb reveals that the heme is partially exposed to solvent,[1] whereas the heme in *Tt* H-NOX is buried within the protein matrix (Figure 1).

The ability of phosphorescent molecules to sense O_2 is determined by the degree of emission quenching in the presence of O_2. Comparison of the steady-state emission spectra of Ru *Tt* H-NOX and Ru Mb measured under aerobic and anaerobic conditions reveals that O_2 appreciably quenches the emission of both proteins (Figure 2). To further evaluate the highly stable Ru *Tt* H-NOX protein as an O_2 sensor, its excited state lifetime was measured at several O_2 concentrations (Figure 3). The data were analyzed according to the Stern−Volmer (SV) equation for O_2 quenching (Supporting Information) and yielded a bimolecular quenching constant, k_q, of 1350 $mmHg^{-1}$ s^{-1} (8.2×10^8 M^{-1} s^{-1}). In addition to intrinsic emission properties, the precision of lifetime-based O_2 sensors is governed by the instrument error associated with the lifetime measurement. Taking our instrument error of 2.5% into account, Ru *Tt* H-NOX can be used to determine O_2 concentrations to within ±2.5 mmHg (4.2 μM) in spite of its low quantum yield. This precision is comparable to that reported for commercial O_2 sensors (Table 2) and is ideally suited for determining O_2 concentrations in biology. Indeed, emission quenching was observed to be linear across the biologically relevant range of O_2 concentrations (Figure 3).[9]

The RuMP proteins described here represent a new class of sensors for detection of dissolved O_2. The sensors are readily expressed in *E. coli*, exceptionally robust, and able to detect O_2 levels in the biologically relevant range. The photophysical properties may be further modulated with the choice of emissive porphyrin or through modification of the protein scaffold (e.g., via site-directed mutagenesis). In addition, the proteins may be expressed with genetically encoded tags for targeted delivery in biology and derivatized for enhancing biocompatibility. We anticipate that this new class of sensors will prove useful for monitoring O_2 levels in biological contexts. One area of particular interest for sensing O_2 is in tumor microenvironments wherein detailed knowledge of local O_2 concentrations is key to improving cancer diagnosis and treatment.[9]

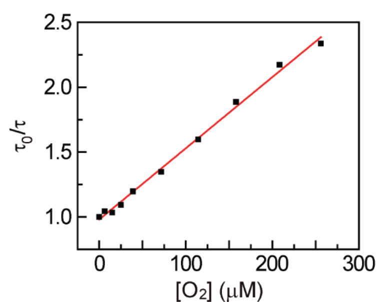

Figure 3. Stern−Volmer plot of the excited state lifetime of Ru *Tt* H-NOX vs [O_2] showing linear phosphorescence quenching by O_2 from 0 to 256 μM O_2 (R^2 = 0.9957).

Table 2. Comparison of Ru *Tt* H-NOX with Select O_2 Sensors

Complex	k_q ($mmHg^{-1}$ s^{-1})	τ_0 (μs)	Precision[a] (mmHg)	Ref
Ru *Tt* H-NOX	1350	7.7	5.0	this work
$Ru^{II}(bpy)_3^{2+}$	4300	0.58	21	16
Oxyphor	293	707	0.25	17
PtP-C343	150	60	5.9	12

[a] Determined assuming an error of 2.5% in τ_0 measurement.

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Supporting Information Available: Synthetic details, porphyrin stochiometry, further photophysical characterization of the RuMP proteins, structural alignments, and X-ray diffraction data. This material is available free of charge via the Internet at http://pubs.acs.org.

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