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Direct interfacial Y₇₃₁ oxidation in α_2 by a photo β_2 subunit of *E. coli* class Ia ribonucleotide reductase†

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Proton-coupled electron transfer (PCET) is a fundamental mechanism important in a wide range of biological processes including the universal reaction catalysed by ribonucleotide reductases (RNRs) in making *de novo*, the building blocks required for DNA replication and repair. These enzymes catalyse the conversion of nucleoside diphosphates (NDPs) to deoxynucleoside diphosphates (dNDPs). In the class Ia RNRs, NDP reduction involves a tyrosyl radical mediated oxidation occurring over 35 Å across the interface of the two required subunits (β_2 and α_2) involving multiple PCET steps and the conserved tyrosine triad [Y₃₅₆(β_2)–Y₇₃₁(α_2)–Y₇₃₀(α_2)]. We report the synthesis of an active photochemical RNR (photoRNR) complex in which a Re(I)-tricarbonyl phenanthroline ([Re]) photooxidant is attached site-specifically to the Cys in the Y₃₅₆C-(β_2) subunit and an ionizable, 2,3,5-trifluorotyrosine (2,3,5-F₃Y) is incorporated in place of Y₇₃₁ in α_2 . This intersubunit PCET pathway is investigated by ns laser spectroscopy on [Re₃₅₆]- β_2 :2,3,5-F₃Y₇₃₁- α_2 in the presence of substrate, CDP, and effector, ATP. This experiment has allowed analysis of the photoinjection of a radical into α_2 from β_2 in the absence of the interfacial Y₃₅₆ residue. The system is competent for light-dependent substrate turnover. Time-resolved emission experiments reveal an intimate dependence of the rate of radical injection on the protonation state at position Y₇₃₁(α_2), which in turn highlights the importance of a well-coordinated proton exit channel involving the key residues, Y₃₅₆ and Y₇₃₁, at the subunit interface.

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Introduction

Enzymatic control of coupled proton and electron transfer^{1–4} is critical in managing biological processes ranging from energy storage (photosystem II)^{5–9} and conversion (cytochrome c oxidase)¹⁰ to the synthesis of DNA precursors (ribonucleotide reductase).^{11–14} To better understand biological PCET, we have undertaken studies of this process in class Ia RNRs, which catalyse the conversion of nucleoside diphosphates (NDPs) to deoxynucleoside diphosphates (dNDPs)—a process required for synthesis and repair of DNA in all organisms.^{15,16} Catalysis by the class I RNRs proceeds by a radical mechanism requiring coupling of radical transport over 35 Å involving PCET across the two subunits to substrate turnover. The long distance, reversibility, and rate-limiting conformational gating of radical transport have made study of this process challenging. To overcome this challenge, we have developed two

methodologies: photoRNRs^{17–21} and site-specific incorporation of unnatural amino acids in place of pathway residues.^{22–24}

E. coli class Ia RNR has served as the paradigm for this long distance radical transport. It is composed of two homodimeric subunits: α_2 and β_2 . A docking model for this complex,²⁵ substantiated by recent biochemical and biophysical studies,^{26,27} has provided the working model for the radical transport pathway shown in Fig. 1. The active site for NDP reduction resides in α_2 , where the cysteine radical (C₄₃₉•) must be transiently generated during each turnover by the essential diferric-tyrosyl radical (Y₁₂₂•) cofactor in β_2 . This long range oxidation requires a multi-step radical hopping mechanism that involves a specific pathway including four tyrosines (Y₁₂₂ and Y₃₅₆ in β_2 ; Y₇₃₁ and Y₇₃₀ in α_2)^{11,28} and potentially W₄₈ in β_2 .¹¹

Recent attention has focused on the detection of the proposed transient radical intermediates and identification of the operative PCET mechanism at each site. Mössbauer studies have established that Y₁₂₂• reduction in β_2 is triggered by binding of substrate and effector to α_2 ²⁹ and involves proton donation from the water at Fe₁ (Fig. 1). This process involves orthogonal PCET wherein the proton and electron come from different residues. High-field electron paramagnetic resonance (Hf EPR) and deuterium electron nuclear double resonance (ENDOR) have provided atomic level resolution of local hydrogen bond interactions, specifically the co-linearity of the

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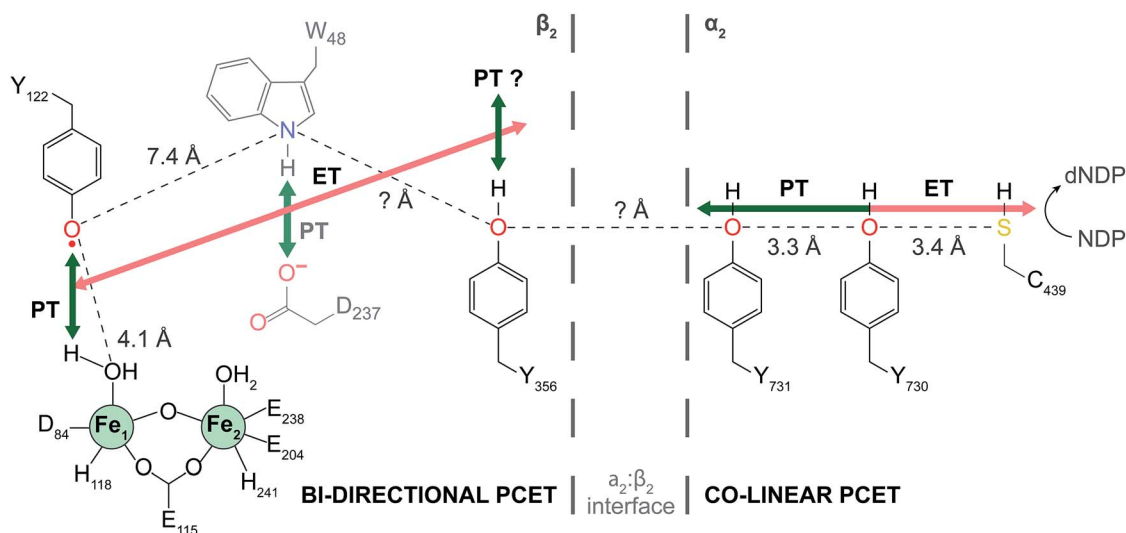


Fig. 1 Current model of radical transport pathway in class Ia RNR leading to nucleotide reduction as determined by the docking model¹⁶ and diagonal distance measurements acquired by PELDOR spectroscopy.³⁴ Key redox active amino acids and known distance measurements involved in PCET pathway are shown. Residue W₄₈ is grayed indicating the absence of experimental evidence supporting its participation in the PCET radical mechanism. Residue Y₃₅₆ is shown at the interface for illustrative purposes. Salmon arrows indicate electron transfer (ET) and green arrows represent proton transfer (PT). In α_2 , co-linear PCET is denoted by the dual-colored arrow, and the proposed bi-directional PCET in β_2 is indicated by the orthogonality of the ET and PT pathways.

PCET within α_2 . Additionally, significant shifts in g_x values together with the assignment of hyperfine coupling features from the ENDOR spectra of various amino-substituted RNR mutants propose an important role for electrostatics at the α_2 : β_2 interface.³⁰ However, the disordered C-terminal tail of β_2 where Y₃₅₆ resides has made interrogation of the chemistry at the subunit interface challenging (Fig. 1).

Rate limiting conformational gating in RNR obscures radical transport across the subunit interface, prompting us to develop photoRNRs to trigger radical initiation with light to avoid this gating and to potentially enable the observation of Y[•] at the interface. Radical injection kinetics were initially made possible using a 19mer peptide photoRNR, which corresponded to the identical 19 residues of the C-terminal tail of β_2 along with a modification that appended a photooxidant (rhenium phenanthroline [Re]) adjacent to Y₃₅₆ or fluorinated derivatives. This peptide photoRNR enables nucleotide reduction in the presence of α_2 and light and allows for observation of radical injection into α_2 .²¹ Radical injection was only realized in the presence of an intact Y₇₃₁–Y₇₃₀ dyad within α_2 , providing important support for co-linear PCET within this subunit.

More recently, photoRNRs have been generated in which the peptide with the photooxidant is replaced by the full-length β_2 containing a site-specifically incorporated [Re] photooxidant at residue 355 using a S₃₅₅C- β_2 mutant.³¹ Transient absorption spectroscopy on the active [Re₃₅₅]- β_2 : α_2 complex in comparison to the control [Re₃₅₅]-Y₃₅₆F- β_2 : α_2 complex allowed for the assignment of a photogenerated [•]Y₃₅₆.³² In addition, further modification of [Re₃₅₅]- β_2 by installation of an unnatural 2,3,5-F₃Y in place of Y₃₅₆ in β_2 yielded the first direct measurement of [•]Y propagation kinetics through the active RNR complex.³³ Comparison of [•]Y decay transient kinetics in the [Re₃₅₅]-2,3,5-F₃Y₃₅₆- β_2 : α_2 complex in the presence of substrate,

either [3'-¹H]-CDP or [3'-²H]-CDP, and effector ATP, revealed an observed rate constant of $1.4 \times 10^4 \text{ s}^{-1}$ and unmasked for the first time an isotope effect on cleavage of the 3' C–H of the substrate. The photoRNRs thus circumvent the masking of radicals by conformational gating and thus have provided insight regarding radical transport and nucleotide reduction chemistry not accessible by any other method.

In this work, the [Re] photooxidant is attached to Cys in the Y₃₅₆C- β_2 mutant and this modified [Re₃₅₆]- β_2 subunit is associated to α_2 in which Y₇₃₁ is site-specifically replaced with 2,3,5-F₃Y. This [Re₃₅₆]- β_2 :2,3,5-F₃Y₇₃₁- α_2 complex together with the [Re₃₅₆]- β_2 :wt- α_2 and [Re₃₅₆]- β_2 :Y₇₃₁F- α_2 control complexes are studied in the presence of CDP and ATP. In contrast to previous photoRNR systems, installation of [Re] at position 356 enables direct interfacial generation of a tyrosyl radical at position 731. Additionally, by leveraging the greater acidity of 2,3,5-F₃Y to enable deprotonation at neutral pH, this residue furnishes an ionizable reporter that varies with experimental pH. In turn, site-selective removal of a single proton at position Y₇₃₁(α) provides the first protein:protein scaffold of RNR that permits the investigation of the effect of a modified proton microenvironment on radical transport on transient time scales (sub μ s) at the interface. In the absence of Y₃₅₆, radical injection is only achieved when position Y₇₃₁ is deprotonated. In addition to confirming the complexity of RNR in maintaining a well-organized PCET pathway, this work introduces and highlights the importance of a well-defined proton exit channel out of α_2 involving the key pathway residues, Y₃₅₆ and Y₇₃₁, at the subunit interface.

Experimental

Modified RNR subunits were constructed, expressed, purified, modified, and characterized as previously reported or with



minor modification.^{19,23,24,31,35} Protein concentrations were measured by absorbance at 280 nm using: $\epsilon_{\alpha_2} = 189\,000\text{ M}^{-1}\text{ cm}^{-1}$, $\epsilon_{\beta_2\text{-apo}} = 121\,000\text{ M}^{-1}\text{ cm}^{-1}$, $\epsilon_{\beta_2\text{-holo}} = 131\,000\text{ M}^{-1}\text{ cm}^{-1}$, and $\epsilon_{\beta_2[\text{Re}]} = 189\,000\text{ M}^{-1}\text{ cm}^{-1}$. Purity of protein constructs was assessed by SDS-PAGE (Fig. S1†). All measurements were conducted in assay buffer at pH 7.6 (50 mM HEPES, 15 MgSO_4 , 1 mM EDTA; unless otherwise specified). Measurement of the dissociation constant (K_D) between $[\text{Re}_{356}]\text{-}\beta_2$ and wt- α_2 was performed by a spectrophotometric competitive inhibition assay as previously reported.^{17,21} Measurement of the pK_a of the phenolic proton of 2,3,5- F_3Y_{731} within the assembled 2,3,5- $\text{F}_3\text{Y}_{731}\text{-}\alpha_2\text{-}[\text{Re}_{356}]\text{-}\beta_2$ complex was performed by fluorometric titration as previously reported.²¹ The details of methods that deviate from published procedures are provided in the ESI.† Similarly, photoinitiated nucleotide reduction activity assays were performed according to published methods.^{17–19,32} Error bars represent 2σ resulting from photolysis on $\geq$ three independent samples.

Time-resolved spectroscopic measurements were performed using a home-built nanosecond laser system previously described.^{21,31–33} Each sample was prepared prior to photolysis and measurements were performed in triplicate. The calculation of the uncertainty in experimental measurements to 95% confidence limits (2σ) is described in the ESI (eqn S1–S5†).

Results and discussion

PhotoRNRs: $[\text{Re}_{356}]\text{-}\beta_2\text{:}\alpha_2$ and $[\text{Re}_{356}]\text{-}\beta_2\text{:}2,3,5\text{-F}_3\text{Y}_{731}\text{-}\alpha_2$

To probe radical initiation across the $\alpha_2\text{:}\beta_2$ interface, specific variants of each subunit were required. To directly target the intersubunit radical transport step of Y_{731} oxidation and subsequent radical injection into α_2 , we chose to circumvent Y_{356} oxidation entirely. In contrast to previous systems where photooxidants were placed adjacent to Y_{356} at position 355, $[\text{Re}]$ replaces Y_{356} in this study. The new construct maintains the mutations C_{268}S and C_{305}S , and preserves catalytic activity. Additionally, the mutation, Y_{356}C , thus enables alkylation with $[\text{Re}]\text{-Br}$ to yield $[\text{Re}_{356}]\text{-}\beta_2$. To examine the effect of a proton at position Y_{731} , this residue was replaced with 2,3,5- F_3Y , solution $pK_a = 6.4$, compared with Y ($pK_a = \sim 10$). The photoRNR construct is illustrated in Fig. 2. These two subunit modifications allow for direct oxidization of Y_{731} by a photo β_2 .

Construction, expression, isolation, and labelling to generate $[\text{Re}_{356}]\text{-}\beta_2$ were performed as previously reported with minor modifications.³¹ Unlabelled and reconstituted $\text{Y}_{356}\text{C-}\beta_2$ (holo) is inactive (0.16(5) U) towards nucleotide reduction (wt- β_2 activity = 6000–8000 U), as expected given the absence of Y_{356} . We note that labelling does not preclude binding ($K_D = 0.43(11)\text{ }\mu\text{M}$), as measured in the competitive inhibition assay shown in Fig. S2.† This value is in agreement with previously reported values for active photoRNR β_2 mutants,³² and not significantly altered from wt-RNR.^{12,36}

$[\text{Re}_{356}]\text{-}\beta_2$ displays similar spectroscopic and photophysical properties, as reported previously for $[\text{Re}_{355}]\text{-}\beta_2$.³¹ Fig. S3† shows typical absorption and emission spectra for $[\text{Re}_{356}]\text{-}\beta_2$; the absorption spectrum is dominated by the characteristic features of rhenium(i) tricarbonyl bisimine compounds and the

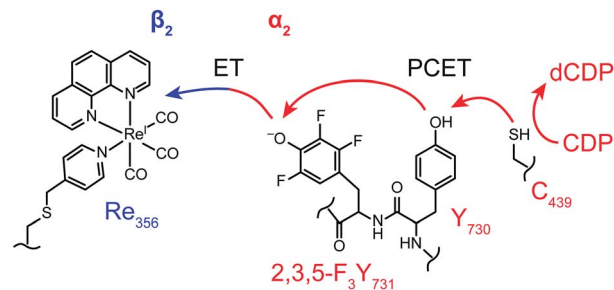


Fig. 2 PhotoRNR design schematic depicting the intersubunit photochemical oxidation of 2,3,5- F_3Y_{731} by $[\text{Re}_{356}]\text{-}\beta_2$, incorporation of 2,3,5- F_3Y at position Y_{731} allowed radical generation to be examined in the absence of a proton at this residue.

emission originates from a triplet metal-to-ligand charge transfer state. The absorption spectrum of $[\text{Re}_{356}]\text{-}\beta_2$ is accurately re-constructed as the sum of $\text{Y}_{356}\text{C-}\beta_2$ and twice the $[\text{Re}]\text{-Br}$ absorption spectrum, as expected for a construct with two $[\text{Re}]$ molecules per β_2 dimer (Fig. S3†).

Construction of 2,3,5- $\text{F}_3\text{Y}_{731}\text{-}\alpha_2$ was achieved using *in vivo* nonsense suppression methodology previously developed to install fluorotyrosine reporters in class Ia RNR.²⁴ The specific activity of 2,3,5- $\text{F}_3\text{Y}_{731}\text{-}\alpha_2$ under catalytic conditions (375(5) U) is diminished relative to wt- α_2 (1800–2500 U), while under single-turnover activity (2.75(4) equiv. dCDP/ α_2) is comparable to wt- α_2 (~ 3 dCDP equiv./ α_2). This decrease in catalytic activity, though comparable to that of wild-type (wt), is consistent with previous reports of this mutant.³⁷

The deprotonation of 2,3,5- F_3Y_{731} is expected to perturb the rate of radical generation at position Y_{731} . The kinetic penalty associated with proton transfer is alleviated by removal of the proton when experimental pH $> pK_a$, thus requiring measurement of the precise pK_a of 2,3,5- F_3Y_{731} in the assembled construct. As previously reported,^{19–21,32,33,38} the increase in the rate of photooxidation for tyrosinate relative to tyrosine,³⁹ makes $[\text{Re}]$ emission a reporter of the protonation state of nearby tyrosine residues. Fluorometric titration of the $[\text{Re}_{356}]\text{-}\beta_2\text{:}2,3,5\text{-F}_3\text{Y}_{731}\text{-}\alpha_2$ complex reveals the pK_a of 2,3,5- F_3Y_{731} to be 6.7(1), Fig. 3. Accordingly, $\sim 90\%$ of 2,3,5- F_3Y_{731} residues are deprotonated under experimental conditions at the optimal operating pH for RNR (pH 7.6). Given the thermodynamically unfavourable acidity of the tyrosyl radical cation ($pK_a = -2$) a PCET process managing both the electron and proton transfers is mandated.^{40,41}

Photoinitiated substrate turnover

To establish that the photoRNR construct is competent to generate dCDP, the $[\text{Re}_{356}]\text{-}\beta_2\text{:}2,3,5\text{-F}_3\text{Y}_{731}\text{-}\alpha_2$ complex in the presence of $[\text{H}^3]\text{-CDP}$ and ATP was photolyzed for 10 min ($\lambda > 313\text{ nm}$) and dCDP was measured by scintillation counting. The results of this single turnover experiment are shown in Fig. 4. Perturbation of the enzyme by the introduction of $[\text{Re}]$ results in a reduced level of turnover that is 5–10% relative to wt-RNR under the same pH conditions. Notwithstanding, the presence of photogenerated products establish the relevance of



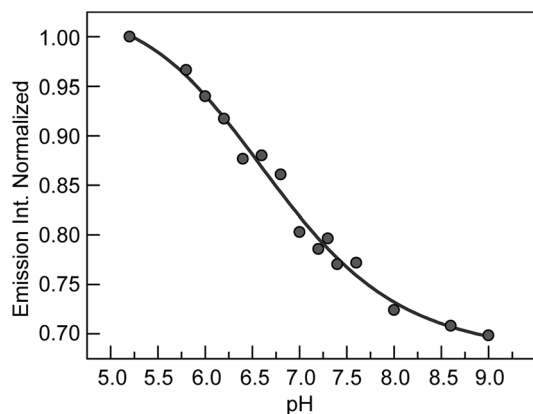


Fig. 3 Measurement of the pK_a of the phenolic proton of 2,3,5- F_3Y_{731} in the $[Re_{356}]\text{-}\beta_2\text{:}2,3,5\text{-}F_3Y_{731}\text{-}\alpha_2$ complex by steady-state emission. Fluorometric titrations of 2.5 μM 2,3,5- $F_3Y_{731}\text{-}\alpha_2$, 3.75 μM $[Re_{356}]\text{-}\beta_2$, 1 mM CDP, and 3 mM ATP were carried over a pH range of 5 to 9. Samples were illuminated with $\lambda_{exc} = 315$ nm, scanned from 420–700 nm at 0.5 nm intervals, integrated for 1 s at each data point, and averaged from three scans. The collected emission plots were integrated for fluorescence intensity and plotted against pH. Data were fit to an internal sigmoidal logistic function in OriginPro 8.5. The inflection point of the monoprotic pH-titration curve (x_0) is the pK_a : $y = A2 + \frac{A1 - A2}{1 + \left(\frac{x}{x_0}\right)^p}$.

$[Re_{356}]\text{-}\beta_2\text{:}2,3,5\text{-}F_3Y_{731}\text{-}\alpha_2$ complex to the natural enzyme. Attenuated enzymatic activity is also detected at $pH > 7.6$ which is consistent with the observed pH rate profiles for the wt enzyme and fluorotyrosine derivatized RNR constructs.⁴²

Radical injection kinetics

The photogeneration of product for the $[Re_{356}]\text{-}\beta_2\text{:}2,3,5\text{-}F_3Y_{731}\text{-}\alpha_2$ complex prompted us to undertake radical injection kinetics

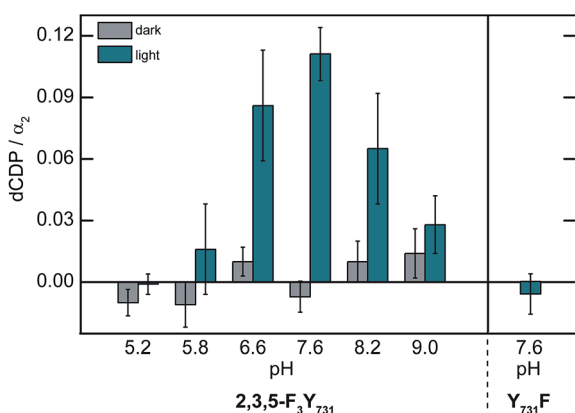


Fig. 4 Photoinitiated nucleotide reduction assay monitored (left) as a function of pH in the $[Re_{356}]\text{-}\beta_2\text{:}2,3,5\text{-}F_3Y_{731}\text{-}\alpha_2$ complex and (right) at $pH = 7.6$ for the control, $[Re_{356}]\text{-}\beta_2\text{:}Y_{731}F\text{-}\alpha_2$. A solution containing 25 μM $[Re_{356}]\text{-}\beta_2$, 200 μM $[^3H]\text{-CDP}$ (21 148 cpm nmol^{-1}), 3 mM ATP in assay buffer, and a separate solution of 10 μM α_2 were incubated at 25 °C. Following two min incubation the two solutions were mixed, transferred to the photolysis cuvette, and illuminated ($\lambda > 313$ nm) for 10 min.

Table 1 Radical injection rates from $[Re_{356}]\text{-}\beta_2$ to α_2 variants modified at position 731 at $pH = 7.6$

$[Re_{356}]\text{-}\beta_2\text{:} X_{731}\text{-}\alpha_2$	$\tau_{600 \text{ nm ns}^{-1}}^a$	$k_q/10^4 \text{ s}^{-1b}$
F	652(4)	—
Y	629(4)	5.6 (1.4)
2,3,5-F_3Y	593(6)	15.4 (2.2)

^a Lifetime of emission decay measured on 10 μM $[Re_{356}]\text{-}\beta_2$, 25 μM α_2 (as indicated), 1 mM CDP, 3 mM ATP in assay buffer ($pH 7.6$), $\lambda_{exc} = 355$ nm, $\lambda_{obs} = 600$ nm. Errors shown in parentheses represent 2σ resulting from measurement on ≥ 3 independent samples. ^b Emission quenching rate constant, k_q , determined from eqn (1). Error in quenching rate constants calculated as shown in ESI.

studies. Using the $[Re]^*$ emission lifetime as a reporter for radical injection, ns TA laser spectroscopy on the $[Re_{356}]\text{-}\beta_2\text{:}\alpha_2$ complexes in the presence of CDP and ATP was conducted. The emission decay lifetimes for each construct were measured at $pH = 7.6$, where maximum turnover was observed, and are summarized in Table 1; representative traces are included in Fig. S4.† As previously observed for $[Re_{355}]\text{-}\beta_2$,³² the $[Re]^*$ lifetime in $[Re_{356}]\text{-}\beta_2$ ($\tau = 507(3)$ ns) increases upon binding to $Y_{731}F\text{-}\alpha_2$ ($\tau_0 = 652(4)$ ns), consistent with the more hydrophobic environment engendered by the protein environment. The lifetime τ_0 of the $Y_{731}F\text{-}\alpha_2$ variant provides a reference for excited-state decay of $[Re]^*$ when it is located at the interface, but in the absence of quenching by the tyrosine located at position 731. Upon introduction of Y_{731} , the $[Re]^*$ emission ($\tau = 629(4)$ ns) is quenched relative to $Y_{731}F\text{-}\alpha_2$ according to the following equation:

$$k_q = \frac{1}{\tau} - \frac{1}{\tau_0} \quad (1)$$

Accordingly, this quenching rate constant, k_q , is equivalent to the radical generation rate, and from eqn (1) it is calculated to

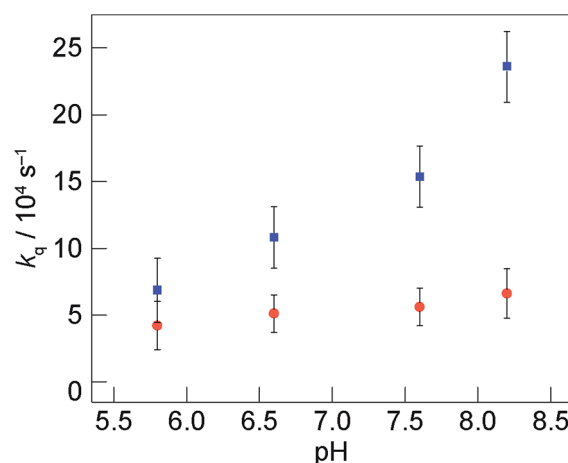
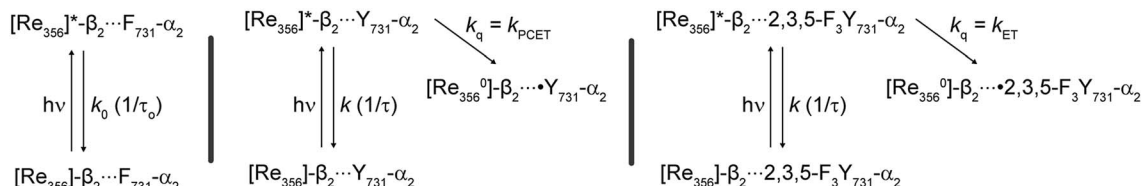


Fig. 5 Quenching rate constant of $[Re]^*$ by tyrosine, k_q , monitored as function of pH: $Y_{731}(\text{wt})$ relative to $Y_{731}F$ (●) and 2,3,5- F_3Y_{731} relative to $Y_{731}F$ (●). τ_0 corresponds to the lifetime of $[Re_{356}]\text{-}\beta_2\text{:}Y_{731}F\text{-}\alpha_2$. Error bars in k_q represent 2σ resulting from error propagation on lifetime measurements as described in ESI.†





Scheme 1 Excited-state deactivation pathways for $[\text{Re}_{356}]\text{-}\beta_2$ in the presence of indicated α_2 subunit.

be $5.6(1.4) \times 10^4 \text{ s}^{-1}$. In comparison to previously observed photooxidation of Y_{356} in $[\text{Re}_{355}]\text{-}\beta_2$ ($k_q = 4.1(1) \times 10^5 \text{ s}^{-1}$), the direct oxidation of Y_{731} by $[\text{Re}_{356}]\text{-}\beta_2$ is slower possibly owing to an increased charge transfer distance, which is occurring across the subunit interface *vs.* at adjacent positions in previous investigations. Further analysis of the emission decay kinetics of $[\text{Re}]^*$ reveal a dependency on the rate of radical injection and the protonation state of tyrosine at position $\text{Y}_{731}(\alpha)$. Replacement of Y_{731} with 2,3,5- F_3Y_{731} enhances the quenching rate constant by a factor of three ($\tau = 593(7) \text{ ns}$ ($k_q = 15.4(2.2) \times 10^4 \text{ s}^{-1}$). This acceleration of radical injection into α_2 is in accordance with the differing protonation states of tyrosine in the two constructs. Under the experimental conditions of $\text{pH} = 7.6$, 2,3,5- F_3Y_{731} is deprotonated and hence quenching occurs by ET rather than PCET, which results in faster tyrosine oxidation, despite being $\sim 50\text{--}100 \text{ mV}$ more difficult to oxidize than native tyrosine at this pH (as determined from solution peak potentials (E_p) of N-acetyl and C-amide protected fluorotyrosines measured by differential pulse voltammetry).²² For clarity, obtaining precise single residue midpoint potentials in protein constructs is extremely challenging, and thus thermodynamic considerations must be guided from these measured E_p values,¹¹ despite recent findings that suggest significant deviations of the formal reduction potential and solution E_p values.⁴³

To investigate how the proton at position Y_{731} affects interfacial PCET and subsequent interfacial radical injection kinetics, emission quenching of the $[\text{Re}]^*$ within the $[\text{Re}_{356}]\text{-}\beta_2\text{:}2,3,5\text{-F}_3\text{Y}_{731}\text{-}\alpha_2$ complex was monitored over the activity accessible pH region of RNR. A plot of the $[\text{Re}]^*$ decay lifetimes for $[\text{Re}_{356}]\text{-}\beta_2$ alone and in the three $[\text{Re}_{356}]\text{-}\beta_2\text{:}\alpha_2$ variant complexes monitored as a function of pH are shown in Fig. S5;† representative emission decay traces are provided in Fig. S4.† The quenching rate constants, k_q , as a function of pH may be determined from eqn (1); these rate constants are plotted in Fig. 5. Quenching by $\text{Y}_{731}(\text{wt})\text{-}\alpha_2$ (red circles, ●) and 2,3,5- $\text{F}_3\text{Y}_{731}\text{-}\alpha_2$ (light blue squares, ■) are referenced to the control, $\text{Y}_{731}\text{F}\text{-}\alpha_2$. Within our error limits, little to no dependence of k_q is observed for the $[\text{Re}_{356}]\text{-}\beta_2\text{:}\text{Y}_{731}(\text{wt})\text{-}\alpha_2$ complex (●), as native tyrosine is protonated throughout the pH window. Conversely, a large pH dependence is observed for k_q when $[\text{Re}_{356}]\text{-}\beta_2\text{:}2,3,5\text{-F}_3\text{Y}_{731}\text{-}\alpha_2$ is compared to Y_{731}F (■). Guided by our measurement of the pK_a of 2,3,5- $\text{F}_3\text{Y}_{731}\text{-}\alpha_2$, shown in Fig. 3, we ascribe the observed differences in quenching to the relative ratio of deprotonated:protonated forms of 2,3,5- F_3Y_{731} as pH is varied. The large k_q s at high pH is expected owing to deprotonation of F_3Y_{731} whereas quenching at low pH approaches that of Y_{731} where both of the tyrosines are protonated.

Scheme 1 summarizes the decay pathways for $[\text{Re}]^*$ in the different variants. The presence of tyrosine introduces an excited-state decay pathway *via* radical generation. For the case of $\text{Y}_{731}(\text{wt})\text{-}\alpha_2$, oxidation occurs by PCET whereas for 2,3,5- $\text{F}_3\text{Y}_{731}\text{-}\alpha_2$ occurs by ET. These differing mechanisms of tyrosine oxidation for the two variants in the absence of Y_{356} introduce the possibility that Y_{356} may be involved with facilitating proton removal at the interface, though future experiments are needed to establish its specific role. While a co-linear $\text{Y}_{356}\text{-Y}_{731}$ π -stacked mode where Y_{356} acts directly as the proton acceptor for Y_{731} is unlikely in light of recent Hf EPR/ENDOR data on amino-substituted tyrosine derivatives at various pathway positions, the strongly perturbed g_x values of $\text{NH}_2\text{Y}_{356}^{\cdot-}$ indicate that Y_{356} may communicate with Y_{731} through a network of water molecules.³⁰ Additional evidence supporting the involvement of Y_{356} in modulating Y_{731} oxidation was also observed in $[\text{Re}_{355}]\text{-}\beta_2$ construct.³² The photooxidation kinetics of Y_{731} , which are summarized in Table S1,† indicate that Y_{731} radical generation is enhanced by the presence of Y_{356} as $[\text{Re}_{355}]\text{-}\beta_2$ oxidation of Y_{731} is 2.1(1.2) times faster for Y_{356} than for F_{356} . The presence of Y_{356} may facilitate proton removal from α_2 *via* the interface, thus assisting in PCET.

In this directed study, whereby Y_{356} is absent by virtue of its replacement with $[\text{Re}_{356}]$, efficient injection of a radical into α_2 is realized only when a proton is removed from the pathway by the introduction of 2,3,5- F_3Y_{731} . While this result does not implicate Y_{356} directly as the proton acceptor for Y_{731} , it supports the contention that Y_{356} is in communication with Y_{731} at the $\alpha_2\text{:}\beta_2$ subunit interface and that Y_{356} enables the PCET required for efficient radical transport. Further investigations of this contention are underway along with studies to assess the role of possible contributions from other residues, or perhaps metal ions that may also be involved in managing protons at the interface.

Conclusions

Replacement of Y_{356} by a $[\text{Re}]$ photooxidant and installation of 2,3,5- F_3Y at position 731 in α_2 furnishes a photoRNR that specifically targets intersubunit radical transport. This construct supports photoinitiated substrate turnover, confirming its fidelity to the natural system. Time-resolved emission studies reveal that 2,3,5- F_3Y_{731} is oxidized at a rate 3 times faster than native Y_{731} even though the non-natural amino acid is thermodynamically more difficult to oxidize at $\text{pH} 7.6$ ($\Delta E_p \sim 50\text{--}100 \text{ mV}$). These results emphasize the enzymatic imperative for coupling the proton and electron to allow for efficient



radical transport. In conjunction with the parallel studies of $[\text{Re}_{355}]\text{-}\beta_2$, these results suggest the importance of a well-coordinated proton exit channel involving Y_{356} and Y_{731} as key interfacial residues for radical transport across the $\alpha_2:\beta_2$ interface.

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