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Proton-coupled electron hopping in Ru-modified *P. aeruginosa* azurin

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Abstract

We constructed two artificial multiple-step electron transfer (hopping) systems based on *Pseudomonas aeruginosa* azurin where a tyrosine (YOH) is situated between Ru(2,2'-bipyridine)₂(imidazole)(histidine) and the native copper site: RuH107YOH109 and RuH124-YOH122. We investigated the rates of Cu^I oxidation by flash-quench generated Ru^{III} over a range of conditions that probed the role of proton-coupled oxidation/reduction of YOH in the reaction. Rates of Cu^I oxidation were enhanced over single-step electron transfer by factors between 3 and 80, depending on specific scaffold and buffer conditions.

1. Introduction

Proton-coupled electron transfer (PCET) reactions play central roles in many biological redox processes. The broadest definition of PCET includes all net transformations in which both protons and electrons are transferred, regardless of mechanism.¹ Many redox processes involve PCET, including conversion of NAD⁺ to NADH, H⁺ to H₂, and reduction of CO₂ to carbohydrates. The phenol side chain of tyrosine (YOH) is known to participate in the PCET reactions of photosystem II,² ribonucleotide reductase,³ and cytochrome *c* oxidase.⁴ Herein, we demonstrate that proton-coupled multiple-step redox reactions (hopping) via YOH can facilitate long-range oxidation of Cu^I in Ru-modified *Pseudomonas aeruginosa* azurin. We regret that we did not have a chance to share our findings with Bob Williams, most especially since his groundbreaking work over many years greatly influenced our views of the reactivity patterns of blue copper proteins.⁵

PCET reactions involving a single proton and electron can occur by any one of three limiting mechanisms: sequential electron transfer followed by proton transfer (ETPT) or vice versa (PTET), or concerted transfer of both particles (CPET).⁶ Investigations of PCET mechanisms in the above enzymes, and in small molecule models, have generated lively debate about the relative importance of each reaction pathway.^{7,8,9,10,11} Reactions of model compounds in non-aqueous solvents typically favor concerted (H• transfer) mechanisms, which avoid the generation of high-energy (charged) intermediates.¹² Conversely, reactions

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Dedicated to the memory of Robert J. P. (Bob) Williams.

of model complexes in water can take advantage of both stepwise and concerted mechanisms, especially PTET, since aqueous PT reactions are facile.^{7,10,11}

The redox reactions of phenol (PhOH) serve as models for biological oxidations of YOH. Oxidation of phenols¹³ and related models¹⁴ H-bonded to proton acceptors in polar, aprotic solvents (e.g., acetonitrile) is thought to occur via a concerted mechanism. In buffered aqueous media, oxidation of PhOH proceeds via PTET.¹¹ It is thought that buffer conjugate bases can act as proton acceptors under some conditions,^{9,11} a role played by H₂O in unbuffered media. Indeed, at certain pH values, H₂O is thought to serve as proton acceptor in concerted oxidation of PhOH to PhO[•].¹¹

Incorporation of phenol (as YOH) into a protein matrix can have a dramatic impact on the energetics and kinetics of its redox reactions. Work on peptides¹⁵ and proteins¹⁶ has shown how local interactions modulate YOH redox behavior, and investigations of modified ribonucleotide reductases¹⁷ have shed light on the roles of YOH reduction potentials in redox function. In adding to this body of work, we have investigated two-step YOH-promoted ET in Ru-labeled azurins (Figure 1) where YOH is situated between a histidine-ligated Ru^{II}(bpy)₂(im) photosensitizer: RuH107YOH109 and RuH124YOH122 (bpy = 2,2'-bipyridine, im - imidazole). We previously demonstrated that these two proteins have cofactor arrangements that are spatially optimized for ET hopping.¹⁸

2. Results

2.1 Synthesis and characterization

All *P. aeruginosa* azurins were expressed and purified as described previously.^{18,19} Note that we used azurins that contain only one YOH residue; all of the YOH and W residues in wild-type azurin were replaced with F and the native H83 is Q. The Ru-labeling protocol is described elsewhere.¹⁹ The labeled proteins were characterized using mass spectrometry (see Supporting Information) and UV-vis spectroscopy.

2.2 pK_a determination

We evaluated the pK_as of YOH109 and YOH122 by monitoring absorbance changes (295 nm²⁰) as a function of pH. Titrations were carried out using 20 μM Cu^{II} azurin in 250 mM sodium borate + 100 mM NaCl (pH values of 7.94, 9.07, 10.02, 11.14, 12.34, and 12.81). Data were fit as described in the experimental section. As the pH increased, a new band appeared at 295 nm, indicative of YO⁻ formation. Analysis of this band gave pK_a(YOH109) = 10.2 ± 0.3 and pK_a(YOH122) = 10.5 ± 0.1. The shift in pK_a between YOH109 and YOH122 is consistent with our findings for nitrotyrosines at the same positions.¹⁸ The titration data showed isosbestic points for conversion of YOH to YO⁻, but the optical band for Cu^{II} shifted above pH ~11, suggesting perturbation of the metal site (the protein may be partially unfolded at high pH values).

2.2 Time-resolved laser spectroscopy

We explored YOH-promoted hopping in the Ru-azurins using standard laser flash-quench techniques. Transient absorption at 632.8 nm monitors the Cu oxidation state; increased

632.8 nm absorption is consistent with the formation of Cu^{II} azurin by reaction with photochemically generated $\text{Ru}^{\text{III}}(\text{bpy})_2(\text{im})(\text{His})$.

2.3 pH dependence with imidazole buffer

Cu^{I} oxidation was probed in RuH124YOH122 (Figure 2) and RuH107YOH109 (Figure 3) at different pH values ($[\text{im}] = 250 \text{ mM}$; $[\text{NaCl}] = 100 \text{ mM}$). Cu^{I} oxidation was not observed at pH 5 for RuH107YOH109. For all other samples, Cu^{I} oxidation kinetics were fit to a single exponential model; the observed rate constants are set out in Table 1. The specific rates of Cu^{I} oxidation by Ru^{III} were enhanced over single-step ET by factors between 3 and 82, depending on the protein and pH. The greatest enhancement was for RuH107YOH109 at pH 9; the smallest enhancement was found for RuH124YOH122 at pH 5.

2.4 Buffer concentration

We also investigated the effects of buffer concentration on ET kinetics in the Ru-azurins. The range of imidazole buffer concentrations was limited, owing to the difficulty of maintaining a fixed pH in solutions where $[\text{imidazole}]_{\text{total}} < [\text{Ru}^{\text{III}}(\text{NH}_3)_6\text{Cl}_3]$. Cu^{I} oxidation kinetics were examined for RuH124YOH122 and RuH107YOH109 proteins using 250, 100, and 10 mM imidazole + 100 mM NaCl at pH 8. Kinetics traces are shown in Supporting Information. In the absence of buffer (pH adjusted with NaOH/HCl), we did not observe oxidation of Cu^{I} in RuH107YOH109 azurin; and the rate of oxidation of Cu^{I} in RuH124YOH122 is within experimental error of that in the RuH124K122 protein.²¹

2.5 Buffer identity

To probe further the role of buffer in YOH-mediated Cu^{I} oxidation, we also explored ET reactions using Tris [tris(hydroxymethyl)aminomethane], sodium borate, and sodium acetate (all at 250 mM + 100 mM NaCl, pH 8). Kinetics traces are shown in Supporting Information and the observed rate constants are set out in Table 3. The observed rate constants were essentially independent of the specific buffer. However, in the case of RuH124YOH122 in acetate ($\text{p}K_{\text{a}} = 4.76$), the kinetics deviated somewhat from single exponential, and the rate constants were smaller than those for ET reactions in the other buffers. The differences in behavior of the two proteins could be due to the different environments surrounding YOH122 and YOH109.¹⁸

3. Discussion

3.1 Cu^{I} oxidation

Oxidation of Cu^{I} by flash-quench generated $[\text{Ru}(\text{bpy})_2(\text{im})(\text{HisX})]^{3+}$ was accelerated over single-step ET in the proteins we investigated. In analogy to findings from work on 3-nitrotyrosine-assisted¹⁸ and Trp-assisted ET,²² we attribute the rate enhancements to multistep tunneling made possible by the introduction of YOH between Ru^{III} and Cu^{I} sites. The pH dependence of the hopping rate enhancements suggests that proton movement associated with YOH oxidation plays a critical role. Involvement with buffer also is suggested, as ET rate enhancement over single-step Cu^{I} to Ru^{III} ET was not observed in the absence of buffer.

3.2 Mechanism

The YOH-mediated oxidation of Cu^{I} by Ru^{III} could occur by any one of several mechanisms. In the following discussion, we focus on the first ET step ($\text{YOH} \rightarrow \text{Ru}^{\text{III}}$), as we did not observe tyrosyl radicals, indicating very rapid $\text{Cu}^{\text{I}} \rightarrow \text{YOH}$ ET. First, the oxidation of Cu^{I} by pure ET hopping (via a $\text{YOH}^{+/0}$ couple) can be eliminated on thermodynamic grounds, because the driving force ($-G^\circ$) for such a reaction is near -0.4 eV ($E^\circ(\text{Ru}^{\text{III/II}}) = 1.0$ V;²³ $E^\circ(\text{YOH}^{+/0}) = 1.4$ V^{6b} versus NHE). In addition, the pK_a of YOH is too high to be consistent with the population of tyrosinate (YO^-) required for mediation of hopping. Hopping maps (Figure 4) constructed as described previously²⁴ support these predictions.

Three remaining potential mechanisms for the initial oxidation of YOH by Ru^{III} are ETPT, PTET, and CPET, where oxidation of YOH occurs with concerted H^+ transfer to buffer or to water. Involvement of YOH^{*+} (the first intermediate in the ETPT pathway) is unlikely, owing to the highly endergonic initial ET step (*vide supra*). Concerted reactions where H_2O is the H^+ acceptor¹¹ also are unlikely, given the limited reactivity without added buffer. Under our experimental conditions, this pathway is not competitive with recombination between Ru^{III} and reduced quencher (0.1-1 ms).

A concerted mechanism, with buffer as the H^+ acceptor, also is not favored. First, marked changes in the kinetics would be expected upon buffer concentration changes. The observed yields of Cu^{I} oxidation products did not change and the rate constants were only marginally perturbed. Using previously derived values for free tyrosine,^{9b} we expect that the fraction of azurin-YOH hydrogen bonded to buffer (imidazole in this case) would go from $\sim 90\%$ (250 mM buffer) to $\sim 20\%$ (10 mM buffer). However, the binding concentrations of YOH/buffer could differ markedly from those reported by Meyer and Thorp^{9b} given the protein environment surrounding the YOH residue. Similarly, changing the identity of the buffer will change the driving force of a concerted reaction, which should be reflected in the rate constants. We did not observe behavior consistent with this prediction; the only exception was minimal perturbation of RuH124YOH122 in acetate buffer, where the pH is far from the buffer pK_a value. Finally, we emphasize that concerted oxidation of phenols in model systems is associated with substantial nuclear reorganization.¹⁴

It is difficult to simulate the kinetics of the reaction, likely owing to uncertainties in YOH and YO^- reduction potentials as well as ET distances in the protein, or the explicit buffer effect. Buffer presence is a requirement for ET rate enhancement over single-step ET, suggesting a buffer-mediated reaction. However, little dependence on buffer concentration or on buffer pK_a on ET rate constants was observed (a larger ET rate dependence on buffer characteristics was expected). These results could mean that buffer is bound under all experimental conditions tested. For the concentrations we have studied, a binding constant of only 10^3 M^{-1} would be needed to be consistent with negligible change in the fraction of buffer bound to the protein. Although there is room for other interpretations, we suggest, given the dependence of Cu oxidation on pH, coupled with the exergonic ET step from the phenolate and the requirement of buffer, that PTET oxidation of YOH accounts for the Cu^{I} to Ru^{III} ET rates.

4. Conclusions

We demonstrated that a tyrosine residue situated between Ru^{III} and Cu^I redox sites in azurin accelerates copper oxidation by up to a factor of 80. The rates depend upon solution pH, buffer, and to a lesser extent the identity of the buffer. Analysis using hopping maps suggests that the first ET step (oxidation of YOH by Ru^{III}) controls the overall rates of Cu^I oxidation. For this first step, our results are inconsistent with a concerted mechanism, where YOH is oxidized and deprotonated in a single step. Instead, our data suggest that a stepwise pathway (proton transfer to form YO⁻, followed by oxidation) is operative. Such stepwise pathways may well occur during the biological redox reactions of tyrosine.

5. Materials and Methods

Buffer salts and solvents were obtained from J. T. Baker. Imidazole was from Sigma-Aldrich. Terrific broth was from BD Biosciences. Solutions were prepared using 18 MΩ-cm water, unless otherwise noted. Ru(2,2'-bipyridine)₂Cl₂ and [Ru(NH₃)₆]Cl₃ were from Strem Chemicals. Ru(NH₃)₆Cl₃ was recrystallized prior to use.²⁵ Mass spectrometry was performed in the Caltech Protein/Peptide MicroAnalytical Laboratory (PPMAL).²⁶ UV-visible spectra were recorded on an Agilent 8453 diode array spectrophotometer. All data were collected at ambient temperature (~293 K).

Plasmids encoding for mutant azurins were generated using the Stratagene Quikchange protocol. Proteins were expressed and purified using known protocols. Purity was assessed using UV-vis and mass spectrometry.

The pK_as of YOH109 and YOH124 residues were determined by measuring optical spectra of solutions prepared by addition of aliquots of a concentrated azurin solution (in water) to a buffer solution of sodium borate (250 mM, pH 8-13) and NaCl (100 mM). Spectroscopic isosbestic points for each titration are consistent with just two absorbing species (YOH and YO⁻). As YO⁻ is the only species absorbing at 295 nm, pK_as values could be extracted from fits of the pH-dependent absorbance (*A*_{295 nm}) to Eqn. 1 (α is a pH-independent constant).

$$A_{295nm} = \frac{\alpha \times 10^{pH - pK_a}}{1 + 10^{pH - pK_a}} \quad (1)$$

All transient spectroscopic measurements were conducted in the Beckman Institute Laser Resource Center at Caltech. Excitation (500 nm) was provided by an optical parametric oscillator (Spectra-Physics, Quanta-Ray MOPO-700) pumped by the third-harmonic of a Q-switched Nd:YAG laser (Spectra-Physics, Quanta-Ray PRO-Series, 8 ns pulse width), as described elsewhere.²⁷ All experiments, which were carried out at ambient temperature (295K), employed [Ru(NH₃)₆]³⁺ (12 mM) as quencher, with 35 μM labeled azurin. Kinetics traces were collected at 632.8 and 490 nm for each protein sample. Protein samples were reduced using sodium ascorbate and desalted using PD-10 columns (GE Healthcare) into the appropriate buffer solution. The samples were deoxygenated by repeated pump-backfill cycles and left under an argon atmosphere for data collection. Data were fit to single exponential functions (Eqn. 2, see Supporting Information).

$$\Delta A_{632nm} = (\Delta A_0 - \Delta A_\infty)e^{-kt} + \Delta A_\infty \quad (2)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Models of RuH107YOH109 (left) and RuH124YOH122 (right) azurins. The structures are based on those determined for rhenium-modified azurins (PDB ID 1I53 and 2I7S, respectively). The site of Ru-coordination is shown with a teal sphere and the site of YOH incorporation is highlighted in red. For clarity, only the protein backbone connecting the histidine and Cu-ligating residue is shown.

Figure 2. Transient absorption traces (632.8 nm) for Cu^I oxidation in RuH107YOH109 azurin. The fits (red solid lines) are single exponential functions.

Figure 3. Transient absorption traces (632.8 nm) for Cu^{I} oxidation in RuH124YOH122 azurin. The fits (red solid lines) are single exponential functions.

Figure 4.

Hopping maps for pure electron transfer reactions. The parameters used to construct the maps are shown on the right. The numbers 1, 2, and 3 refer to Ru^{III}, YOH, and Cu^I respectively. The dotted black line represents the driving force for the Ru^{III} – Cu^I → Ru^{II} – Cu^{II} reaction. Hopping is not predicted to occur via YOH (upper black dot) in either of the proteins. Hopping is predicted to occur via YO⁻ with rates of 4.3×10^5 and 1.5×10^6 s⁻¹ for RuH107YOH109 and RuH124YOH122, respectively. The dashed line is drawn at 0.7 eV.

Table 1

Cu^I oxidation rate constants (k_{obs} , s⁻¹) at different pH values.

pH	RuH107YOH109	RuH124YOH122
single-step ^a	$(2.4 \pm 0.5) \times 10^2$	$(2.2 \pm 0.2) \times 10^4$
5	---	$(6.7 \pm 0.2) \times 10^4$
6	$(5.7 \pm 0.3) \times 10^3$	$(8.0 \pm 0.2) \times 10^4$
7	$(1.2 \pm 0.2) \times 10^4$	$(8.5 \pm 0.2) \times 10^4$
8	$(1.7 \pm 0.2) \times 10^4$	$(1.0 \pm 0.2) \times 10^5$
9	$(1.8 \pm 0.2) \times 10^4$	$(1.4 \pm 0.2) \times 10^5$

^aRef. 21. Single-step data for proteins without YOH (M109 and K122) Data were collected in 100 mM sodium phosphate, pH 7. For RuH107, [Co(NH₃)₅Cl]Cl₂ was used as an irreversible quencher and the rate constant was extrapolated to infinite dilution of RuH107-azurin.^{21c,d,e}

Table 2

Rate constants at different buffer concentrations (+100 mM NaCl, pH 8).

[buffer] (M)	RuH107YOH109	RuH124YOH122
0.25	$(1.7 \pm 0.2) \times 10^4$	$(1.0 \pm 0.2) \times 10^5$
0.1	$(1.5 \pm 0.2) \times 10^4$	$(1.0 \pm 0.2) \times 10^5$
0.01	$(1.3 \pm 0.2) \times 10^4$	$(9.8 \pm 0.2) \times 10^4$

Table 3
Rate constants in different buffers (+100 mM NaCl, pH 8)

buffer	p <i>K</i> _a	RuH107YOH109	RuH124YOH122
acetate	4.76	$(1.3 \pm 0.3) \times 10^4$	$(5.0 \pm 0.5) \times 10^4$
imidazole	7.05	$(1.7 \pm 0.2) \times 10^4$	$(1.0 \pm 0.2) \times 10^5$
Tris	8.07	$(1.7 \pm 0.2) \times 10^4$	$(1.6 \pm 0.2) \times 10^5$
borate	9.14	$(1.8 \pm 0.2) \times 10^4$	$(1.7 \pm 0.2) \times 10^5$